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**Serum autoantibodies as tumour markers in breast cancer:**  
**their role in screening, diagnosis and prognosis**

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Dedicated to:

Mimi

Senada

Mum

Dad

God

*In the name of God, the merciful, the beneficent.*

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ABBREVIATIONS:

AAb	Autoantibody
AAbs	Autoantibodies
ABC	Advanced Breast Cancer
ADH	Atypical Ductal Hyperplasia
ALH	Atypical Lobular Hyperplasia
AMA	American Medical Association
APC	Antigen Presenting Cell
ATAC	Adjuvant Tamoxifen <i>versus</i> Arimidex <i>versus</i> Combined Study
CA 27-29	Cancer Antigen 27-29
CA15.3	Cancer Antigen 15.3
CC	Cranio-caudal
cDNA	Complementary DNA
CEA	Carcinoembryonic Antigen
CI	Confidence Interval
CIC	Circulating Immune Complex
CMF	Cyclophosphamide Methotrexate 5` Fluorouracil
CR	Coefficient of Reproducibility
CT	Computer Tomogram
CV	Coefficient of Variability

DAB	Diaminobenzidin
DCIS	Ductal Carcinoma In-Situ
DFI	Disease Free Interval
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dPCR	Differential Polymerase Chain Reaction
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked ImmunoSorbant Assay
ESR	Erythrocyte Sedimentation Rate
ER	Oestrogen Receptor
fhx	Family history
Fig.	Figure
FISH	Fluorescence In-situ Hybridisation
GIVIO	Interdisciplinary Group for Cancer Care Evaluated
GPs	General Practitioners
HER2	Human Epidermal Growth Factor Receptor 2
HRP	Horse Radish Peroxide
HRT	Hormone Replacement Therapy
H <sub>2</sub> O <sub>2</sub>	Hydrogen Peroxide

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
KS	Kolmogorov-Smirnov
LCIS	Lobular Carcinoma In Situ
LN	Lymph Node
MAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MLO	Medio-lateral Oblique
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
N	Number
N/A	Not Available
NHSBSP	National Health Service Breast Screening Programme
NICE	National Institute of Clinical Health and Excellence
NIH	National Institute of Health
NPI	Nottingham Prognostic Index
NS	Non significant
NSS	Normal Swine Serum
NST	Non-specific Type



OD	Optical Density
OS	Overall Survival
PBC	Primary Breast Cancer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEM	Polymorphic epithelial mucin
PET	Positron Emission Tomogram
PSA	Prostate Specific Antigen
PVP	Polyvinyl Pyrrolidone
SD	Standard Deviation
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SELDI	Surface-enhanced Laser Desorption/Ionization
SLE	Systemic Lupus Erythematosus
StreptABComplex	Streptavidin Biotin Complex
TBS	Tris Buffered Saline
TMB	3,3',5,5' -tetramethyl-benzidine
TPA	Tissue Polypeptide Antigen
Tween	Polyoxyethylenesorbitan monolaurate
UICC	International Union Against Cancer
USA	United States of America

VI	Vascular Invasion
VNTR	Variable Number Tandem Repeat
VOL	Vector Only Lysate
WHO	World Health Organisation
+ve	Positive
-ve	Negative
%	Percent

## **Abstract**

### **INTRODUCTION:**

Early diagnosis of breast cancer can result in less radical therapy and improved survival. Current screening and diagnostic tools have limitations, as do serum marker antigens due to their low sensitivity. We hypothesised that an immune response is an early event in cancer evolution. Autoantibodies, which are the amplified signals of cancer-derived antigens, can be detected in the peripheral blood of women with early breast cancer. This thesis is a continuation of previous work at the Nottingham Breast Unit aimed at developing new panel of assays for the detection of autoantibodies in breast cancer. The goal of this thesis was to investigate the use of a potentially more reproducible ELISA assay to measure serum autoantibodies to MUC1, p53 and c-myc either singly or in combination within a panel to further clarify a role of AAbs in screening, diagnosis or prognosis of primary breast cancer.

### **METHODS:**

Newly expressed, biotinylated and reconfigured p53 and c-myc antigens and purified MUC1 antigen were used to establish novel in-house ELISA. These were used to measure autoantibodies to the above 3 antigens in the serum of various populations which were collected over a two year period. These populations included an at-risk population (e.g. family history and atypical ductal hyperplasia) and a population of women who had just been diagnosed with primary breast cancer, either non-invasive ductal carcinoma in situ (DCIS) or invasive cancers. Cut-off values were established for each of the

autoantibodies based on 2 or 3 standard deviations from the mean of a population of control samples. The control samples were obtained from a population of women who were either deemed 'normal' or who had a histological diagnosis of benign breast disease. The assay was validated by assessing effect of sample age as samples were of varying age, reproducibility using Bland Altman coefficient of reproducibility and reliability by establishing the assays ability to distinguish cancer from non-cancer.

## RESULTS:

Eight hundred and ninety eight samples were analysed in the study. One hundred and ten were Control samples. The remaining samples included 381 that were from an at-risk population and 407 that were from a primary breast cancer population. Mean ages of Control, at-risk and primary breast cancer populations were 58.8, 50 and 62.9 years respectively.

Data establishing validity of assay confirmed that sample age did not affect signal strength for MUC1 and c-myc autoantibodies. Older samples for the p53 autoantibody had lower signal than recent ones. Reproducibility data was satisfactory and was best in the samples from the group of women with benign breast disease. Using either a 2 or 3 standard deviation cut-off value the assay was also able to distinguish cancer from non-cancer for both MUC1 and p53 autoantibodies. For the c-myc autoantibody, cancer samples showed increased signal compared to non-cancer although this did not reach significance.

The at-risk population were routinely followed up in an outpatient clinic dedicated for women at increased risk of breast cancer. An individual positive marker was noted in up to 10% of at-risk patients. The panel of 3 assays showed a raised marker in 18.4%. This was significantly higher than that for the Control population whose panel detection was 9.1% whilst an individual marker was noted in up to 4.5% of samples. Only the c-myc autoantibody had similar prevalence in both Control and at-risk populations. There was no correlation between risk category and autoantibody detection.

The specificity for MUC1, p53 and c-myc autoantibody serum tumour markers were 92.4%, 95.2% and 95% respectively. Specificity of the assay can be further increased if two or more markers were needed to be positive before a positive result is deemed for the assay.

Thirteen women in the at-risk group developed breast cancer. The panel had a higher sensitivity to detect occult tumours compared to individual markers but at reduced specificity. Two of 13 at-risk patients (15.4%) who developed breast cancer had a raised marker (MUC1 & p53 autoantibodies) within the panel with a mean lead-time of 43.5 months. Further increasing the cut-off value to Mean + 4 standard deviation of Control population increased the specificity of the panel assay to 97.2% without altering the sensitivity to detect occult tumour (15.4%).

Primary breast cancer population consisted of patients who were known to have DCIS or invasive breast cancer. The latter group was further subdivided into those who were detected via screening mammogram (screen-detected) and those who presented with a lump (symptomatic). Two of the 3 markers (p53 and c-myc autoantibodies) were

significantly raised in the primary breast cancer population compared to the at-risk population as well as the Control group as detailed in earlier paragraph. Individual markers were detected in up to 20.9%, 10.3% and 9.8% for p53, c-myc and MUC1 autoantibodies respectively. The panel detection rate was 35.1%.

The tumour markers showed limited use as a prognostic factor. Only the c-myc autoantibody correlated with a poorer survival due to distant metastasis in symptomatic breast cancers. Data for the screen-detected breast cancer cases showed that there were no correlation between any of the 3 serum marker detection and prognosis.

#### CONCLUSION:

Our data demonstrated the three autoantibody assays whether singly or in combination as a panel showed differences not only between cancer and non-cancer but also between Control and at-risk, as well as between at-risk and cancer.

The panel showed that one or more assays were positive in 35% of breast cancers with a specificity of 83.6%. The specificity of the assay can be altered to meet clinical needs by either increasing the cut-off value or altering the markers within the panel. Current data in the literature suggests a number of markers that may be added or substituted into the panel to enhance the specificity and sensitivity. However a sensitivity of 15.4% for detection of occult tumour in the at-risk group makes any clinical application for screening in this group less cost effective using the version of the assays described in this thesis. The lead-time in the two patients who did show elevation of an autoantibody

suggests that if the sensitivity and specificity can be improved that there is an in-vivo amplification signal, which might allow earlier identification of some breast cancers.

Detection of c-myc autoantibodies indicates a poorer prognosis in the symptomatic group. The value of this information needs to be further determined in larger studies and within multivariate analysis. If the current results remain then there may be clinical implication to this early data.

Comparison with previous data from the unit revealed that detection of cancer-associated autoantibodies in primary breast cancer and at-risk groups using this methodology appeared to be less sensitive. This may indicate that the current method has been successful in reducing background signal and hence reduce false positive results. It therefore appears that we have established a more reliable and reproducible assay compared to previous study to detect autoantibodies to tumour-associated antigens. However it is noted that this thesis reports single batches of antigens (MUC1, p53 and c-myc) used in the autoantibody assays. Investigation of differences in protein structure and immunogenicity between batches, which might also affect the sensitivity and specificity of these assays, was outside the scope of this thesis but is the subject of ongoing research by other members of the research group.

**Serum autoantibodies as tumour markers in breast cancer: their  
role in screening, diagnosis and prognosis.**



# Chapter 1

## Breast Cancer Screening

## 1.1. CURRENT BREAST CANCER SCREENING

Current methods of diagnosis rely on the patient's own symptomatic detection or detection by routine mammographic surveillance. Worldwide experience of breast screening via mammography has been generally successful. Screening has been shown to reduce breast cancer mortality by around 25% in the screened population (Tabar et al 1989; Tabar et al 2001; Tabar et al 2003). The mortality benefits of mammographic screening have been further confirmed both by the WHO International Agency for Research on Cancer committee (WHO handbook, 2002) and Swedish combined trials group (Nystrom et al, 2002).

Mammography detects cancers based on a variety of signs recognised by the radiologist to be associated with a possible neoplasm. Some breast tumours such as lobular carcinomas are not as easily detectable on mammograms and may therefore be more likely to be missed on routine screening (Holland et al, 1983). Some lobular cancers not visible on mammography are however detectable on ultrasound (Watermann et al, 2005), although the latter is not a useful screening tool. It does however have a high sensitivity for symptomatic lesions (Ohta et al, 2005).

Three yearly mammograms in the screening programme in the UK results in a higher number of interval tumours with each succeeding year from the last screening mammogram. These are tumours detected in-between mammograms. Their detection rates rise from 0.58 to 1.53 per 1,000 screened from the 1<sup>st</sup> to 3<sup>rd</sup> year after screening (Raja et al, 2001). Although some data suggest one year between screening mammograms is probably optimal (Smith et al, 2003) current evidence do not indicate any survival advantage to reducing the interval period from every 3 years to one yearly (Breast Screening Frequency Trial Group 2002).

In the UK the screening interval has remained at 3 years although the age for screening has been extended from 64 years to 69 years. This age range is to be further increased to 47-73 years of age by 2012 (<http://www.cancerscreening.nhs.uk/breastscreen/index.html>). In addition since the screening programme was started in the UK, mammography has moved to two view and double reading on the basis that both of these will increase the detection rate (Blanks et al 1998).

Generally breast tumours detected by mammographic screening are smaller and more node negative, thereby leading to a better prognosis (Cowan et al, 1997). It is also believed that many early lymph node negative tumours have microscopic metastasis in the sentinel lymph node, significance of which is still debated (den Bakker et al, 2002).

## 1.2. SENSITIVITY AND SPECIFICITY OF MAMMOGRAMS

Mammography has been the main mode of imaging for breast cancer for more than thirty years. However the sensitivity of mammography is age dependent. This is due to denser breast reducing the ability of mammograms to detect early lesions. As younger women have denser breast then sensitivity of mammograms reduces with younger age. In women above 60 years of age the sensitivity is 95% but this reduces to less than 50% in those 40 years or less (Kolb TM et al 2002; Ashley et al 1989). Specificity of mammograms in symptomatic patients is generally high with reports ranging from 87.7% (Barlow et al, 2002) to 98.6% (Siddhartha et al, 2008). As is the case for sensitivity, the specificity of mammography also reduces in the younger patients with denser breast tissue (Barlow et al, 2002). Furthermore in the screening population, mammogram screening has a high recall rate of 9.8%, although

cancer detection is only 4.7 per 1000 (Rosenberg et al, 2006). Such high recall rates can lead to anxiety and distress to patients.

Due to the differential sensitivity of mammograms for different ages screening recommendation differs around the world. The AMA, the American College of Radiology, American Cancer Society and NIH have all advocated a double projection mammogram yearly for all women aged 40 years or more (U.S. Preventive Services Task Force, 1996).

This is not the consensus in other countries. Evidence suggests mortality benefit of screening is greatest for women aged 55 to 70 years (Nystrom et al, 2002). For women aged 40 – 49 there is only limited evidence of mortality benefit (Miller, 2002). Most countries have an age limit of 65 –70. Recently the Dutch health service has introduced screening to women up to 75 years old. A model analysis has shown that screening up to this age would give a reasonable balance between favourable and unfavourable effects (Fracheboud et al, 2006).

Mammographic sensitivity is also lower in women who have been on HRT for a prolonged period. Oestrogen preparations are believed to increase the density of breast tissue and thereby reduce the sensitivity of mammography (Topal et al, 2006). Interval cancers have been noted to be more common in this group of women compared to HRT never-users in a recent study (Crane et al, 2002).

Despite the above limitations of mammograms, their effectiveness in reducing breast cancer deaths in women between the ages 50-70 years of age is well documented (Tabar et al 1989; Tabar et al 2001; WHO handbook, 2002; Nystrom et al, 2002; Tabar et al 2003; Shen et al. 2005). Cancer related survival in women with screen-detected breast cancer is already greater

than 90% at 5 years (Yassin et al, 2003). Therefore although there may be limitations of mammography as detailed in the above section, adjunctive or alternative screening tools may add very little to survival in this group of women whose prognosis is already excellent.

### 1.3. SCREENING AT- RISK WOMEN

Women are at increased risk either through increased genetic susceptibility or histological risk. The former includes those with known genetic mutation or have significant family history of breast or other cancers but no proven genetic mutation. The latter include those with histologically proven atypical ductal hyperplasia (ADH) or lobular carcinoma in-situ (LCIS). A third group of at-risk include patients previously exposed to mantle radiotherapy for lymphoma. Genetic mutations in BRCA1 and BRCA2 genes account for majority of inherited genetic breast cancers.

The degree of risk in those with family history without a known genetic mutation can be measured using risk stratification models such as Claus (Claus et al 1994) or Gail (Gail et al 1989). Individuals with 4 or more first-degree relatives with breast or ovarian cancer at any age are regarded as high risk. They have a 17% risk of breast cancer within 10 years or 30% lifetime (NICE guidelines 2006). These patients can be screened from a younger age dependent on their risk status as ascertained by these models (NICE guidelines 2006).

Methods of screening the high-risk group who are younger than 40 years of age include magnetic resonance imaging (MRI) (Tilanus-Linthorst et al, 2000; Warner et al, 2001; Kriege et al, 2004). Addition of MRI to mammograms in this group of at-risk population increases the sensitivity from 25-59% for mammogram alone to 93-100% for MRI and mammogram

(Lord et al, 2007). In a recent multicenter trial for comparing the various modalities in surveillance of at-risk women, routine MRI screening was the most sensitive compared to other forms of radiological screening (Sardanelli et al, 2007). Financial restrictions may however prevent its general acceptance. Another disadvantage of MRI, which may limit its use, is a lower specificity compared to other modalities (Riedl et al, 2007). This is supported by a meta-analysis suggesting a 3 to 5-fold increase in recall rate for false positive MRI scans (Lord et al, 2007). There is also difficulty to perform real-time MRI guided biopsies (Elmore et al, 2005).

#### 1. 4. DISEASE BURDEN

Breast cancer is the most common cancer in women and the second leading cause of cancer related death. One woman in 9 will develop the disease in her lifetime and 1 in 29 will die as a direct result of it (Office for National Statistics, 1999). It is estimated that on average a woman who dies of breast cancer will have lost 19.3 years of life that she may have had if she did not develop the disease (Greenall and Wood, 2000). This number is highest in younger women for whom mammography is less useful as a screening modality and who in general appear to present with more aggressive disease (Anders et al 2008). Boyle and colleagues calculated that "41% of the years of life lost due to breast cancer diagnosed before the age of 80 years are attributable to cases presenting symptomatically at ages 35-49 years." (Boyle et al, 1995) The undeniable loss of quality of life and productivity from the time of diagnosis, although difficult to calculate, should also be acknowledged.

## 1. 5. SENSITIVITY AND SPECIFICITY

Tests for screening, diagnosis and monitoring therapy have performance characteristics such as sensitivity and specificity. Sensitivity is defined as the proportion of people with disease who have a positive test result. Specificity is defined as the proportion of people without disease who have a negative test result.

The former is calculated by the formula:

$$\text{sensitivity} = \frac{\text{number of True Positives}}{\text{number of True Positives} + \text{number of False Negatives}}.$$

The latter is calculated by the formula:

$$\text{specificity} = \frac{\text{number of True Negatives}}{\text{number of True Negatives} + \text{number of False Positives}}$$

All tests require both high sensitivity and specificity to be regarded as reliable. Diagnostic tests require high degree of sensitivity; however screening tests particularly require high specificity. Since the denominator for screening is the normal population, any percentage drop in specificity by definition results in a large number of essentially normal people being investigated. This in turn leads to increased medical test and clinical workload with resultant increase in financial cost (Srivastava and Gopal-Srivastava, 2002). Poor specificity also results in great distress to patients who undergo further investigations for false positive results during screening.

## 1. 6. EARLY DETECTION AND TREATMENT

The advance of screening technologies for breast cancer will only be possible if occult tumours can be localised and treated with an overall gain in survival. Recent advances in MRIs allow the detection of smaller lesions not detectable on mammograms (Warner et al, 2008).

Positron emission tomogram (PET) with 18-fluorodeoxyglucose can be used to diagnose and locate early breast cancer (Hayashi et al, 2008). It can also be used to detect early recurrent breast tumours in patients with elevated serial serum tumour markers but clinically silent disease (Aide et al, 2007; Radan et al, 2006). A study group had shown that tumour marker-guided PET scan in the follow-up of breast cancer patients has a sensitivity of 92%, specificity of 75% and a positive predictive value of 89% in the detection of occult tumour recurrence (Suarez et al, 2002). Screening for breast cancer using PET scanning is not however used routinely as the current cost is excessively high. Furthermore for small breast tumours (< 2cm diameter), the sensitivity has been reported to be 48-68% (Yasuda and Ide, 2005), a rate that is prohibitively low for adequate screening.

Treatment in the form of breast conserving surgery may then be directed to the specific tumour. Adjuvant radiotherapy, endocrine therapy or chemotherapy may be directed by the tumour pathology. As smaller tumours are less likely to have positive lymph nodes (Kolias et al 1999; Ma et al 2007), their overall prognosis is expected to be favourable and therefore may prevent the use of chemotherapy.



Pilot studies have shown that early intervention on elevated serum tumour antigen markers have significant implications on the management and outcome of patients with breast cancer with an increase in metastatic free survival and overall survival noted (Jager et al, 1994; Nicolini et al, 1997). It may also be possible that the nature and type of treatment advocated to these patients will be dependent on the specific nature of the positive results i.e. the quality and quantity of tumour marker rise.

Despite such promising possibilities, there is still no general consensus that any earlier detection than current screening mammograms results in survival advantage (Khatcheressian 2008; Jatoi 2005). Although a recent meta-analysis concluded that early detection of loco-regional recurrence did significantly improve survival (Lu et al 2008), early detection in those with advanced disease do not appear to significantly alter the outcome.

## Chapter 2

### Tumour Markers

## 2.1. TUMOUR MARKERS IN GENERAL

### 2.1.1. PRINCIPLES OF TUMOUR MARKERS

As a normal cell transforms to a neoplastic cell, changes occur both within and on the surface of the cell that could potentially be detected and used as a tumour marker. This could provide valuable information on the status of the cell at the given point thus enabling early detection, which is key to cancer cure and prevention (Srinivas et al, 2001). Although there are many modalities available to detect early tumour e.g. computer tomogram (CT) and MRI, laboratory based detection has an added advantage that it is relatively inexpensive. The cost-benefit analysis is favourable for this type of investigation as the unit cost of the test is low and will reduce further with more high-throughput assay innovations (Srivastava and Gopal-Srivastava, 2002).

### 2.1.2. HISTORY OF TUMOUR MARKERS

Current tumour markers are generally tumour associated-antigens. The first tumour marker noted was in 1960s with the description of carcinoembryonic antigen (CEA) (Gold and Freedman, 1965). It was noted that this was present in the serum of patients with gastrointestinal malignancies but not in normal mature tissues. It was hoped that this and other markers would be highly sensitive and specific to the tumour in question. It was thought they could be used not only for diagnosis but also in screening. However it was later realised that the same tumour markers were not only detected in other malignant conditions but were also found in various quantities in normal cells (Thomson, 1972). Their role in distinguishing malignant from benign thus became unclear.

### 2.1.3. CURRENT ROLE OF TUMOUR MARKERS

Current clinical application of tumour markers is limited to diagnosis of recurrent or metastatic disease such as CEA in colorectal cancer and CA15.3 in breast cancer. Tumour markers are also used to monitor response to systemic therapy in certain cancer patients.

If tumour markers are to be clinically applicable for screening or diagnosis then the marker must be present in the serum of the at-risk individual in sufficient quantity and not be present in the normal population. The assays used to measure these markers need to have a high sensitivity and specificity for the detection of markers. The assay also has to be relatively inexpensive and the disease tested is common and causes significant morbidity and/or mortality if left unchecked. A positive assay should result in definitive treatment with survival advantage in those treated compared to the untreated group (Daar and Aluwihare, 2000).

### 2.2. BREAST CANCER TUMOUR MARKERS

Unfortunately breast cancer has yielded no such simple screening blood test to-date. The overwhelming prevalence of breast cancer in the north European and American populations and its morbidity and mortality and some limitations of current screening methods demand a simple and reliable test similar to the prostate specific antigen (PSA).

Tumour markers in breast cancer are extremely various in number and type. Mucins e.g. CA15.3 (Clinton et al, 2003; Safi et al, 1991) and CA 27-29 (Frenette et al, 1994), oncofoetal proteins (e.g. CEA) (Esteban et al, 1994; Sundblad et al, 1996), oncoproteins e.g. HER2

(Imoto et al, 2007; Muller et al, 2006; Kong et al, 2006; Hudelist et al, 2006) c-myc (Breuer et al, 1994). and p53 (Balogh et al, 2006; Hassapoglidou et al, 1993), cytokeratins e.g. TPA (Nicolini et al, 2006; Sliwowska et al 2006) and ESR (Robertson et al, 1991 and 1999; Rubach et al, 1997) are among the many proposed as a tumour marker for breast cancer. More recent tumour markers described in the literature include Mammaglobin (Watson et al, 1996), survivin (Goksel et al, 2007; Yagihashi et al, 2005), livin (Yagihashi et al, 2005), NY-ESO-1 (Bandic et al, 2006), Annexin XI-A (Fernández-Madrid et al, 2006), Endostatin (Balasubramanian et al, 2007), Hsp90 (Pick et al, 2007), p62 (Rolland et al, 2007) and koc (Zhang et al, 2003).

These and other various antigen markers have been used with only limited success. These tumour antigen markers are either over-expressed and therefore produced in excessive amounts or are the mutated form of a corresponding 'wild type'. Normal form and amounts of antigen can also be found but in abnormal compartments of the cancer cell or in extra cellular spaces. Normal functions of these wild type markers vary dependent on the marker and the cell that produces it. However aberrant markers can actually be involved in the pathogenesis of the tumour itself.

### 2.2.1. MEASUREMENT OF BREAST TUMOUR MARKERS

There is a wide range of methods used to test breast tumour markers dependent on the marker itself. These assays include solid matrix-blotting, immunohistochemistry (IHC), fluorescence in-situ hybridisation (FISH), enzyme immunoassay (EIA) and enzyme linked immunosorbant assay (ELISA). The different assays can be used to measure various targets related to the tumour marker, such as DNA or gene copy number (FISH, Southern blot), mRNA (Northern

blot), cell surface protein (Western blot, cell surface ELISA and IHC) and circulating protein (serum ELISA and EIA). Furthermore different tissues can be used depending on the assay used: fresh frozen tissue for Southern, Northern and Western blots and IHC; formalin-fixed, paraffin-embedded tissue for IHC and FISH; and serum or tissue extracts for ELISA and EIA.

There are advantages and disadvantages to using the various methods. For example, IHC is performed using specific antibodies against the tumour marker and depending on the specificity of the antibody IHC may be able to discriminate between normal and abnormal copies of tumour marker and can precisely localize the marker in cells and tissues. However even where an antibody shows promise in distinguishing cancer from normal cells there are many technical issues with IHC such as antigen loss that can occur in stored formalin-fixed, paraffin-embedded tissue samples. This loss is variable and depends on time and nature of fixation; method of tissue processing; temperature of paraffin embedding; duration of storage; the particular antibody used for detection; and the staining procedure used. Therefore variability in results using IHC is partly related to antigen loss as well as use of different antibodies to the same marker.

ELISA can be used to measure breast tumour markers in either fresh tumour cytosolic fractions or in circulating serum as shed antigens or detection of the immune response, as antibodies, to such antigens. The convenience of serum ELISA is that a serum sample can be taken at any time and on repeated occasions whereas tissue samples of primary tumour are usually obtained following biopsy or surgery. Unfortunately one disadvantage of ELISA is that histological information cannot be obtained using ELISA and furthermore an ELISA blood test may measure a different marker endpoint to IHC.

### 2.2.2. BREAST TISSUE TUMOUR MARKERS

Dependent on the marker, these can either be present in cancer cell nucleus, cytosol or the cell membrane. Tissue concentrations of markers may also correspond to tumour load thereby quantifying tumour burden. Depending on the actual marker it may indicate prognosis or predict tumour behaviour.

The expression of some markers such as HER2 within the tumour has been reported to correlate with a rise in serum levels of the marker at an advanced stage of breast cancer (Narita et al, 1994; Molina et al, 1996). Molina and colleagues reported on 200 women treated for primary breast cancers that were followed up with sequential blood samples for measurement of three tumour markers. In 18% of patients the first sign of recurrence in terms of blood antigen measurements was a rise in HER2 in the blood. Further tests showed that serum HER2 was elevated in 80% of patients who were found to be HER2 positive in their primary tumour and in only 3.3% of patients who had a HER2 negative tumour. However this apparent link between tissue and serum antigen expression is not the case for all tumour antigen markers (Cannon et al, 1993).

Tumour marker levels in the tissue cannot therefore accurately predict its presence or level in the serum for the majority of markers and so direct measurement in the serum is necessary. A further disadvantage of measuring tissue markers is that these markers only confer a static view of the tumour. This is in contrast to using serum markers whose detection reflect a dynamic situation and can be repeated as often as required.

### 2.2.3. PROTEOMICS

Tumour markers may be measured singularly or in combination. However, recently whole cell proteins have been measured as biomarkers of cell events (Belhajjame et al, 2005). Since cancer cells have altered oncogene expression, the protein products are also altered. This can be measured as microarrays of multiple proteins.

Individual proteins can also be identified using various techniques such as 2-D gel electrophoresis (Arora et al, 2005) thereby allowing the detection of new single protein tumour marker. This promising field for early tumour detection is aided by various new techniques that detect these proteoms e.g. surface enhanced laser desorption/ionisation (SELDI) (Mazzatti et al, 2007), biochips (Hervas 2004) and mass spectrometer (de Souza et al, 2006).

The measurement of the protein products of oncogenes rather than the genes themselves has several advantages. Proteins are regarded as the dynamic consequence of cellular events and therefore will indicate the cell condition at a given time. Furthermore a single protein translated from a gene may undergo multiple further procedures that can be at fault at any stage and therefore measurement of proteins should be a more accurate reflection of what has gone wrong. This is not possible simply by measuring oncogenes.

The potential uses for proteomics in breast cancer diagnosis, prognosis and monitoring, although undoubtedly huge, are currently undetermined. Although there are many modalities available to detect early tumours e.g. CT and MRI, laboratory-based detection has an added advantage that it is relatively inexpensive (Srivastava and Gopal-Srivastava 2002).



Despite such potential benefits getting results from proteomics takes time. Protein analysis can be laborious. It often requires separating multitude of proteins and determining their individual molecular weight and electric charge. This downside of proteomics may limit its general use. Proteomics may also yield an array of proteins, which are highly specific to the individual patient. It therefore limits general clinical application of the procedure.

### 2.3. SERUM TUMOUR MARKERS

As mentioned in section 2.2 some tumour markers are originally contained within tumour cells. They can be found within the nucleus, cytosol or are membrane bound with extracellular domains.

Nuclear proteins, which can be measured as markers of the cancer, may be sequestered in the cytosol as part of carcinogenesis. Sequestration in an abnormal compartment may prevent the normal function of the cell, thus resulting in eventual carcinogenesis. These intra-cellular markers are released into the serum via non-apoptotic and apoptotic cancer cell death, where they can be measured as serum markers. Other markers may be cleaved from its original membrane bound configuration and shed into the extra cellular domain.

Detection of these serum markers may therefore reflect the overall antigen load of the organism i.e. its cancer burden or the degree of proteolytic activity owing to growth rate, necrosis and cell degeneration.

Serum marker measurement can be by techniques such as ELISA (Cordiano et al, 1995) and SELDI (Mazzatti et al, 2007), which also allow quantification of the marker present. .

SELDI is an affinity-based mass spectrometric method in which proteins of interest are selectively adsorbed to a chemically modified surface on a biochip. Impurities are removed by washing with buffer. These proteins can then be measured on a reader (Li et al, 2002). This therefore allows accurate measurement and profiling of the proteins available in the serum.

Marker levels in the serum are dynamic in that they reflect overall tumour burden as some tumour markers are seen to decrease in level in the serum after excision of the primary tumour. This decrease is not demonstrated in pre and postoperative levels of markers found in non-neoplastic conditions (Reis et al, 2002). This correlation between the markers noted in the serum and actual tumour load is also seen in measuring the parameters of an active extrinsic coagulating pathway. Breast cancer activates this pathway thus resulting in elevated plasma D-dimers that can be directly measured. Elevated plasma levels of this and other markers of the coagulation pathway also correlate with the number of metastatic sites as well as progression kinetics of the tumour (Dirix et al, 2002).

### 2.3.1. SERUM ANTIGENS

As mentioned in section 2.3, serum antigen measurement allows a dynamic overview of disease burden in an individual with respect to progression of the disease as well as response to therapy. Its current clinical role therefore is in the diagnosis of symptomatic metastatic

breast cancer (Robertson et al, 1999 Jan) and also in the monitoring of response during therapy (Murray et al, 1995; Safi et al, 1991; Tondini et al, 1988).

The role of serum antigens in the follow up of patients with primary breast cancer is disputed. GIVIO investigators concluded that intensive follow up of breast cancer patients, clinically and with serum markers did not improve overall survival (GIVIO investigators, 1998). This has also been confirmed by Sato (Sato et al, 2003). Tumour antigen levels in the serum reflect tumour load, therefore in early disease where tumour burden is low, detection of serum antigens as markers can be negligible.

However Molina et al (1995) detected metastases in 40% of those progressing from primary breast cancer to metastatic disease with a lead-time of 4.9 months using serial measurements of serum CA15.3 and CEA. The specificity of these markers for metastatic disease was 99% (Molina et al, 1995).

Data from Nicolini showed that early treatment based on rising tumour markers can result in delaying the onset of symptoms of metastasis (up to 13.5 months) and longer survival compared to those who are treated dependent on onset of symptoms (42.9% vs 13.6% at 30 months) (Nicolini et al, 1997). They concluded that the clinical use of serum antigen markers has advantages over the generally accepted UICC assessment of response (Hayward et al, 1977), which is the preferred method of response assessment in many centres.

It is seen that biochemical progression often occurs ahead of clinical and or radiological progression as the tumour burden required to elicit a positive response in the serum is lower than that required to be noticed radiologically or clinically (Gion.1992). Furthermore disease

stabilization and survival as well as improved quality of life has been noted where marker-directed chemotherapy has been utilised over current method of UICC response assessment. There is also significant cost-savings achieved since earlier discontinuation of expensive chemotherapeutic agents can be directed by tumour marker results.

### 2.3.2. MEASUREMENT OF SERUM ANTIGENS TO DIAGNOSE CANCER

Despite the importance of the measurement of tumour markers in the serum for disease monitoring, the potential use in the diagnosis of cancer is limited. They appear to be neither sufficiently sensitive nor specific for the detection of early breast cancer as they are more a measurement of tumour load. Molina and colleagues reported that only 13% of patients with primary breast cancer had an elevated serum CEA whilst 18.8% had a rise in CA15.3 (Molina et al, 2003).

Using more than one tumour antigen markers in combination may increase the sensitivity but may also result in decreased specificity and their measurement, as a cancer screening tool had yet to be established.

### 2.4. IMMUNE RESPONSE TO CANCER AND ITS USE IN CANCER DETECTION

Malignant transformation of cells is the end result of altered expression of genes that are essential in regulating normal cell growth and differentiation. Oncogenic antigens are the expressed proteins of these altered genes. These gene alterations include both somatic DNA mutation and gene translocation, both resulting in the expression of `foreign` proteins.

An immune reaction to oncogenic proteins has been recognised for sometime with the detection of AAbs to p53 by Crawford in 1982 (Crawford et al, 1982). However the significance of this immunogenic response is not yet understood.

Immune response to cancer cells require the host immune system recognising foreign antigens, which are captured, processed and presented by antigen presenting cells (APCs) to the humoral system via the major histocompatibility complex (MHC) class I and II systems.

It is seen that although oncogenic antigens are contained within numerous cellular compartments they can be shed into extracellular space by enzymatic cleavage or expelled out after tumour-induced necrosis or apoptosis of cells. For this reason AAbs to both intracellular as well as extracellular components of transmembrane receptors have been noted.

Some gene alterations are amplifications rather than mutations thus resulting in over expression of normal proteins. Despite no obvious protein abnormality, the increased availability of protein results in peptides from the protein being presented in higher concentrations by MHC molecules. It therefore renders a non-immunogenic protein immunogenic (Cheever et al, 1995). Thus oncogenic proteins, whether abnormal in structure or quantity can elicit the production of AAbs.

Evidence of humoral immune response can be seen by the isolation of a B-cell producing AAbs against an oncogenic antigen (Polymorphic epithelial mucin (PEM) in this case) in a patient with known ovarian carcinoma (Rughetti et al, 1993). Cellular immune response is also involved as cytotoxic T cells have been shown to recognise oncogenic antigens and

mediate lysis of tumour targets in-vitro (Jerome et al, 1991). The immune response in cancer can therefore be used to aid diagnosis and perhaps also screening.

Measurement of AAbs produced by the humoral immune response may provide an in vivo amplification of tumour antigen markers at an early stage of the disease and therefore provide high sensitivity in terms of early detection. This potential use of AAbs in screening, diagnosis and prognosis of breast cancer is the basis of this present thesis.

#### 2.4.1. ANTIBODIES TO TUMOUR ASSOCIATED ANTIGENS

This thesis is the continuation of work at the Academic Division of Breast Surgery at the University of Nottingham (Cheung 2001). Cheung had studied AAbs to four oncogenic antigens in the serum of several different populations; the at-risk population, the newly diagnosed primary breast cancer population, the normal population and women with benign breast lumps.

The AAbs assayed were against MUC1, p53, c-myc and HER2. Cheung established their role in early diagnosis and treatment in his thesis (Cheung 2001). Longer follow-up data on the at-risk and cancer cases as well as refinement of the assay to reduce background signal were targeted as future development of the original work.

For this thesis, we endeavoured to refine the assay further from the original protocol, thereby reducing background signal and improving reproducibility. The new assay was used to measure the presence of MUC1, p53 and c-myc AAbs in the Normal, primary breast cancer (PBC) and at-risk populations who were followed up longer than previously. We determined

the role of the AAb either as individual or within a panel in screening in this group for cancer detection. Calculation of lead-time, test sensitivity and specificity of the assay in screening and diagnosis was performed. We also endeavoured to assess the potential to use the novel assay in establishing diagnosis and prognosis of primary breast cancer patients who had longer follow-up data than previously.

The HER2 AAb assay was not included in the study, as a reliable and stable HER2 oncoprotein could not be produced during this study. The p53 and c-myc proteins were produced in a bacterial expression vector that allowed the antigens to be expressed in large quantities and produced in a biotinylated form. Biotinylation allowed the oncoproteins to be immobilised onto a neutravidin coated plastic well during ELISA (Cordiano et al, 1995). This form of immobilisation enabled the antigens to be more accessible for AAb binding in the ELISA.

#### 2.4.2 TUMOUR ASSOCIATED ANTIGENS SELECTED FOR THE STUDY

Since breast cancer is a heterogeneous disease, these tumours express many aberrant proteins and there are an increasing number of these described in the current literature and detailed earlier in section 2.2.

Measuring individual markers either as antigens or antibodies gives low sensitivity irrespective of the cancer type or the marker measured in most reported studies due to the heterogeneity of the disease. No single antigen is likely to demonstrate an AAb response in all patients. Two reviews from Zhang demonstrated that combining greater numbers of tumour-associated antigens within a panel will enhance the detection of the specific cancer

using autoantibody assays (Zhang, 2004 and 2007) and different cancers may require different panel of markers (Zhang, 2007). However combination of markers in a panel may decrease the specificity of the panel. Various combinations have been established by different groups and are described in Section 15.1.

Within a panel, combining various mucins has a limited value, as different mucins appear to give equivalent sensitivities (Steger et al, 1989). Since cancer evolution is a multi-step process, it seems reasonable to speculate that choosing markers within a panel that are all formed from different stages will give increased sensitivity than markers from the same stage of carcinogenesis.

Our unit had identified a panel of markers that were involved in various steps of carcinogenesis and could therefore be utilised in screening and early diagnosis of breast cancer. The initial study (Cheung 2001) highlighted four antigens (MUC1, p53, c-myc and HER2) that were present in small amounts in most patients with early disease (Robertson 1990 and 1991a). We have speculated that such small amounts of antigen can induce the production of a larger number of AAbs in the early phase of cancer evolution, which can be detected readily with an ELISA assay employing a novel means of antigen presentation.

In the current body of work, we continued to use MUC1, p53 and c-myc as the antigens within a novel ELISA assay. All three antigens are involved in different cell cycle function and therefore at various steps during carcinogenesis. HER2 antigen was not included in the thesis as a stable and reliable HER2 antigen could not be produced during the study. The use of the above 3 antigens in our study also allowed direct comparison with previous data from our unit and are discussed in length in chapter 3



## **Chapter 3**

### **Tumour Associated Antigens and their Autoantibodies**

### 3.1. MUC1

MUC1 is a glycoprotein that is found in the epithelium of apical surfaces of many 'wet' organs such as bladder (Retz et al, 1998), breast (Croce et al, 1997; Cao et al, 1997), colon (Cao et al, 1997), respiratory tract (Hollingsworth et al 1992) and pancreas (Ho et al, 1993). It is also known as polymorphic epithelial mucin (PEM), CA15.3 antigen and Episialin.

#### 3.1.1. MUC1 STRUCTURE AND ORIGIN

MUC1-mucin is a high molecular weight glycoprotein that exceeds 400KD. It is the encoded product of the MUC1 gene, which is localized to chromosome 1q21–q24. MUC1 is a transmembrane glycoprotein (Kufe et al, 1984). The protein part of this molecule consists of a tandem repeat section consisting of 20 amino acids, to which O linked carbohydrates are attached (Siddiqui et al, 1988). The protein has a variable number of these 20 amino acid tandem repeats (Gendler et al, 1990).

MUC1 protrudes out quite substantially from the cell surface due to the carbohydrate side chains that maintain an extended conformation. Such variations result in a highly variable molecule attained from the MUC1 gene, which, in normal subjects, is found on the apical aspect of epithelial cells in organs such as the breast (Croce et al, 1997; Cao et al, 1997), lung (Hollingsworth et al 1992), colon (Cao et al, 1997) and benign ovarian tumours (Dong et al, 1997). MUC1 is either secreted or shed and can therefore be found in the serum of normal healthy individuals but in low amounts (Bjerner 2002). Its physiological role may be in cell adhesion and cell signalling. MUC1 may also be involved in binding to pathogens and therefore aid protection from these pathogens.

### 3.1.2. MUC1 IN CARCINOGENESIS

Mucin genes encode rod-shaped apomucin cores that then undergo post-translational modification in the cytoplasm by glycosylation thus resulting in the MUC1 glycoprotein. During carcinogenesis, glycosylation of MUC1 is altered due to enzyme deficiencies or increased activity, resulting in an abnormal MUC1, which may then be shed into the lymph and blood circulation (Albrecht et al, 2007). Their presence in this environment allows the monitoring of carcinoma patients.

Due to the wide spectrum of MUC1 in different types of normal healthy cells and thus the vast numbers of different carcinomas which express altered forms of MUC1, many immunoassays employing different monoclonal antibodies are available to detect this rise in MUC1. There is as yet no one gold standard assay and as such these assays retain their corporate names e.g. CA15.3, Bresmarq and CA27.29.

Measurement of MUC1 using the serum CA15.3 assay is now the most widely used serum marker assay for the monitoring of breast cancer, where it can be used to determine metastatic breast cancer recurrence as well as monitoring therapy in those with advanced breast cancer (Molina et al, 1995; Robertson et al, 1999; Murray et al, 1995; Safi et al, 1991; Tondini et al, 1988).

Immunohistochemical staining using anti MUC1 monoclonal antibodies such as CT2 MAb and C595 MAb showed that almost all breast cancer cells are stained mainly in the cytoplasm (93%) and the membrane (73%) (Croce et al, 2003).

### 3.1.3. MUC1 AND PROGNOSIS

Presence of MUC1 in either tissue or serum has been used to ascertain prognosis of breast cancer by many authors (Table 3.1.). Clearly there is no consensus on its prognostic value although more recent papers indicate a poorer disease outcome. Differences in prognosis may be due to the different methods of MUC1 detection as well as differences in study design.

Table 3.1 Literature on MUC1 and prognosis

<b>Study</b>	<b>Method of Detection</b>	<b>Site of Detection</b>	<b>Detection System: Antigen / AAb /mRNA</b>	<b>Prognosis</b>
von Mensdorff-Pouilly 2000	ELISA -	Serum	AAb	Good
Van der Vegt 2007	IHC	Tissue	Antigen	Good
Hermesen 2007	IHC	Tissue	Antigen	Good
Baldus 2005	IHC	Tissue	Antigen	Good
Xu 2001	IHC	Tissue	Antigen	No correlation
De Roos 2007	IHC (DCIS only)	Tissue	Antigen	Poor
Leroy 2006	Review paper	Tissue	Antigen	Poor
Al-Azawi 2006	ELISA -	Serum	Antigen	Poor
Rakha 2005	IHC	Tissue	Antigen	Poor
Nogi 2003	PCR	Bone marrow	mRNA	Poor
Luna-More 2001	IHC	Tissue	Antigen	Poor
Norum 2001	ELISA –	Serum	Antigen	Poor
Cheung 2001	ELISA –	Serum	AAb	Poor
Duffy 2000	ELISA –	Serum	Antigen	Poor
McGuckin 1995	IHC	Tissue	Antigen	Poor

The staining pattern itself can also correlate with prognosis. One study showed a worse prognosis and reduced disease free interval in those with greater than 75% of cells expressing MUC1, especially where the MUC1 staining was cytoplasmic (McGuckin et al, 1995). In a more recent report (Rahn et al, 2001), the staining pattern was confirmed to be mostly cytoplasmic (93% of cells). MUC1 staining was also noted in the apical membrane in 15% and circumferential membrane in 13%, although the majority of tumour cells did appear to have a mixed pattern. This compares to only apical membrane staining in normal and benign cells (Rahn et al, 2001). This study confirmed a poorer prognosis with cytoplasmic staining.

MUC1 antigens were prevalent in non-invasive breast cancer. In this group of early breast cancers, tumours with cytoplasmic staining were more commonly high grade and therefore it was concluded that cytoplasmic MUC1 was an early development in breast carcinogenesis (Diaz et al, 2001).

It has been postulated that since MUC1 is suggested to function in cell adhesion, inappropriate expression during carcinogenesis could result in altered adhesion and therefore aid metastasis. (Segal-Eiras and Croce, 1997).

#### 3.1.4. SERUM MUC1 ANTIGEN

As mentioned in section 3.1.2, serum MUC1 is the most widely measured serum tumour antigen in breast cancer. The MUC1 antigen however can also be measured in the blood of healthy women (McGuckin et al, 1993). Serum MUC1 is also elevated in physiological and non-malignant states such as pregnancy, lactation and infective conditions (Croce et al, 2001). However pregnancy and ovarian cancer-derived MUC1 is morphologically different to

MUC1 from normal tissues (Devine et al 1994). Interestingly, this difference in MUC1 structure is also seen in MUC1 derived from pleural and peritoneal effusions (Yu et al, 2001).

Measuring serum MUC1 is mainly used for the detection of metastatic recurrence of breast cancer and also monitoring therapy in advanced breast cancer (Molina et al, 1995; Robertson et al, 1999; Murray et al, 1995; Safi et al, 1991; Tondini et al, 1988). Elevated serum MUC1 levels are also noted in other types of cancer e.g. ovarian (Wang et al, 2007; Vlad et al 2006) and pancreatic cancers (Nagata et al, 2007; Gold et al, 2005) although no clinical use is established in these cancers.

Serum MUC1 does not correlate with tissue MUC1 expression (Croce, Oct 2003). Its measurement in the serum is dynamic and will correspond to therapy. Our own group reported in 1993 that the expression of antigens in the primary tumour did not correlate with which antigen became elevated in serum at diagnosis of metastases in the same individuals (Cannon et al 1993).

Serum MUC1 can be measured in between 5% - 30% of preoperative breast cancers according to various studies (Arslan et al, 2000; Kokko et al, 2002; Robertson et al, 1990). This variance may be dependent on stage of the disease (Robertson et al 1990). Robertson and colleagues noted only an 8% prevalence of MUC1 in the serum of women with PBC in stage I and II. This rose to 33% when women with stage III cancer was analysed. The variations in sensitivities in different studies may be due to the various assays used to measure MUC1 antigens in the serum. Not all assays are as reliable as one another, with automated assays noted to have the least inter and intra assay coefficient of variation (Bon et al, 1999).

Some authors have reported that a raised level of MUC1 in the serum indicates a poorer prognosis (Kumpulainen et al, 2002; Ebeling et al, 2002; Molina et al, 2003; Duffy et al, 2004; Martin et al, 2006). A persistent rise in serum MUC1 levels above cut-off value following primary chemotherapy for locally advanced breast cancer further indicates reduced disease free interval (DFI) in this group of patients (Al-azawi et al, 2006). One study noted that both tumour stage and pre operative CA15.3 can independently predict survival in PBC patients (Kumpulainen et al, 2002).

A large study on lymph node (LN) negative breast tumours also highlighted a poorer prognosis with patients who have increasing serum levels of MUC1 (Duffy et al, 2004). In this study there does not appear to be any correlation with tumour size, grade, stage and ER status and serum MUC1 levels (Duffy et al, 2004).

MUC1 derived from breast cancer is structurally different from MUC1 derived from normal cells and as a result the former appears to be recognised as ‘non-self’ and induces an immune reaction to this molecule, which can be detected either as free AAbs to various epitopes in the MUC1 molecule or as MUC1-bound circulating immune complexes (CICs).

The immunodominant section of the MUC1 molecule is specific amino acid sequences arising from the variable number tandem repeat section, which has been described in previous paragraphs (Tarp et al 2007). Normal MUC1 protein has these epitopes hidden from the host’s immune system by heavily glycosylated side chains. However, shortened glycan side chains as found in breast cancer, result in the unmasking of these immunodominant regions. These then appear to induce an immune response (Taylor-Papadimitriou 1994).

Another suggested mechanism for the development of an immune response to MUC1 is its prolonged exposure to the immune system in breast cancer patients (Gourevitch et al, 1995).

### 3.1.5. AUTOANTIBODIES TO MUC1

AAbs to MUC1 molecules have been reported to be detected in up to one third of breast cancer patients, in either free or complexed within circulating immune complexes (CIC) (von Mensdorff-Pouilly et al, 1998). These CICs contain both the MUC1 antigen and its autoantibody. IgG is the usual autoantibody found complexed with MUC1 suggesting a specific immune reaction against the MUC1 molecule, although IgM has also been found in 10% of sera from breast carcinoma patients (von Mensdorff-Pouilly et al, 1998). Free AAbs are also noted in pregnant and lactating women although it is IgM that appears more prevalent in the latter circumstance (Croce et al, 2001).

The formation of CICs may account in part for some of the difference noted in MUC1 immunostaining in tissues and the prevalence of the MUC1 antigen in serum; whereas tissue expression of MUC1 is more than 85% (Rakha et al, 2005; Croce et al, 2003), MUC1 is only noted in 10-30% of sera of primary breast cancer patients (Stages 1-3) (Arslan et al, 2000; Kokko et al, 2002; Robertson et al, 1990).

One possible explanation for the above discrepancy is that the autoantibodies that are bound to the MUC1 to form the CIC may mask detection sites within the MUC1 thereby giving falsely low levels of MUC1 in serum (Gourevitch et al, 1995). MUC1-CIC is not measured in current commercial assays and therefore the level of MUC1 within these complexes cannot be determined. The prevalence of MUC-CIC has been reported to range from 25%



(Gourevitch et al, 1995) to 50% of patients with breast cancer (Croce et al, 2003).

The presence of either free MUC1 AAbs or MUC-CIC may signify a better prognosis. This however is only noted in cases where both the MUC1 antigen and the corresponding CIC levels are raised. This suggests that not only is a humoral immune response elicited against cancer but that it may protect against disease progression. However for the protection to be clinically evident it is almost certainly required to be a continuous response. It is on this basis that vaccines against breast cancer, including MUC1 vaccines, are currently being developed (von Mensdorff-Pouilly et al, 1996; 2000).

The aim of this thesis is to measure the levels of free AAbs to MUC1 as a serum tumour marker in the at-risk women to establish its role in screening. Serum MUC1 AAbs were also measured in those presenting with early breast cancer to assess whether as a tumour marker either singly or within a panel of other markers it has any role in diagnosis and prognosis in this group.

### 3.2. P53

p53 is the expressed protein of an oncogene involved in tumour suppression. It plays a pivotal role in regulating DNA repair.

#### 3.2.1 P53 STRUCTURE AND ORIGIN

The p53 tumour suppressor gene is located on chromosome 17p13.1. Its product is a nuclear protein consisting of 393 amino acids and is divided structurally and functionally into four domains. It has an important role in the regulation of growth of both normal and malignant cells.

Two separate p53-mediated mechanisms are known to suppress tumourigenesis; p53-mediated cell cycle arrest and p53-mediated apoptosis (Moll et al, 2001). The latter mechanism is more important in tumour suppression. Although the apoptotic pathway is not fully understood, it is suggested that this oncogene simultaneously engages in a multitude of downstream pathways to mediate cell death (Moll et al, 2001).

Although p53 is a known nuclear protein it can also be localised by IHC to the cellular mitochondria during cell stress and p53 mediated apoptosis (Moll et al, 2001). In p53-mediated cell arrest, cells are blocked near the G1/S border of the cell cycle, thus controlling cell replication.

### 3.2.2. P53 IN CARCINOGENESIS

In many human cancers mutant forms of p53 proteins are present and these mutant p53 gene products no longer suppress cell division. Thirty percent of breast cancers have mutant p53 genes and gene product (Storr et al, 2006 Review). In fact p53 mutations are the single most common genetic change to be characterised in human cancers (Vogelstein et al, 2000). p53 alterations have been reported to play a pivotal role in early breast cancer evolution (Singh et al, 1993). Various mutations of p53 are possible such as a one-base deletion, a two-base deletion, a nine base deletion, point mutation and a complex deletion (Kandioler-Eckersberger et al, 2000).

The mutant form of p53 is more stable than the wild type and therefore has a longer half-life. A second possible mechanism involved in altering p53 in breast cancer has been postulated (Moll et al, 1992). It has been seen that in some breast cancers with wild type p53, the p53, which is normally located in the nucleus, was accumulated in the cytoplasm. The exclusion of the p53 protein from the cell nucleus eliminates the ability of this protein to inhibit the proliferation of cells and therefore inactivates the p53 function independently of mutation (Moll et al, 1992).

### 3.2.3. P53 AND PROGNOSIS

Similar to MUC1, there is controversy in establishing the role of p53 in prognosis of breast cancer. The following table (Table 3.2.) illustrates the various studies analysing prevalence of p53 both as an antigen in tissue and also its antibody in serum. There is no clear consensus whether the presence of p53 in tissue or serum indicates a poorer prognosis.

Table 3.2 Literature on p53 and prognosis

<b>Study</b>	<b>Detection method</b>	<b>Tissue / serum Antigen or AAb detected</b>	<b>Prevalence</b>	<b>Prognosis</b>
Erdem 2005	IHC	Tissue antigen	36.2%	No correlation
Reed 2000	IHC	Tissue antigen	29%	No Correlation
Metcalfe 2000	ELISA	Serum AAb	15%	No correlation
Daliford 1999	ELISA	Serum AAb	7.9%	No correlation
Willsher 1996	ELISA	Serum AAb	48%	No correlation
Regidor 1996	ELISA	Serum AAb	14.5%	No correlation
Sangrajrang 2003	ELISA	Serum AAb	19%	Poor
Gao 2005	ELISA	Serum AAb	21.5%	Poor
Huober 1996	ELISA	Serum AAb	21%	Poor
Lenner 1999	ELISA	Serum AAb	23%	Poor
Mudenda 1994	ELISA	Serum AAb	26%	Poor pathology
Turner 2000	IHC	Tissue antigen	26%	Poor
Silvestrini 1993	IHC	Tissue antigen	44%	Poor

#### 3.2.4. IMMUNOSTAINING OF THE P53 ONCOPROTEIN

The p53 oncoprotein can be detected in the primary tumour by IHC using anti-p53 antibodies such as clone DO7 (Tsutsui et al, 2002) and P180 (Kandioler-Eckersberger et al, 2000). It is usually located in the nucleus. The prevalence of p53 immunostaining in breast cancer tissue ranges greatly from 20% to 50% (Tan et al, 1999; Bartley and Ross, 2002; Tsutsui et al, 2002). This wide variation may be due to the use of different antibodies, staining standards, tumour material, scores for positivity and the inclusion of variously selected groups of breast cancer patients. The presence of p53 in breast cancer tissues have been linked to higher tumour grade and negative oestrogen receptor status (Thor et al, 1992) and a poorer response to conventional treatment with some chemotherapy such as doxorubicin and CMF chemotherapy (Hensel et al, 2000) resulting in a poorer outcome (Elledge et al, 1993).

A recent study by Tsutsui it was noted that the p53 oncoprotein was detected in both primary breast tumour as well as its metastatic lymph node in almost all cases (Tsutsui et al, 2002). It is not known whether distant metastatic disease also immunostains for p53. This would be difficult to establish, as pathological specimens of distant metastasis are usually not available for immunostaining. Correlating the presence of the p53 oncoprotein in tissue and its antibody in the serum is debated. Mudenda had shown good correlation between both tissue and serum p53 (Mudenda et al, 1994) whilst Wilsher demonstrated no such correlation (Wilsher et al, 1996).

### 3.2.5. SERUM P53 ONCOPROTEIN

Currently there is little information on p53 antigen in serum. This is in contrast to data now available on serum p53 AAbs. This therefore makes detection of the p53 antigen in the serum for screening, diagnosis or prognosis difficult to advocate.

### 3.2.6. AUTOANTIBODIES TO P53

Serum AAbs to the p53 oncoprotein were first demonstrated by Crawford in 1982 in 14 of 155 (9%) breast cancer patients using Western Blotting (Crawford et al, 1982; Crawford et al, 1984). Since then other techniques such as ELISA have been used to measure serum p53 AAbs. This provides a more sensitive and rapid assay for these antibodies. A proportion of breast cancers that over-expressed the p53 oncoprotein in their tissues elicited an antibody reaction. The antibody reacted to both over-expressed wild type and mutant form of the p53 (Davidoff et al, 1992) and was directed towards immunodominant epitopes localised in the amino terminus of the p53 protein (Schlichtholz et al, 1994). The presence of AAbs to the p53 oncoprotein and tumour prognosis in breast cancer is still a matter of debate as demonstrated in Table 3.2. Some authors have shown a poorer prognosis whilst others have failed to elicit any prognostic information from serum p53 AAb.

The prevalence of serum p53 AAbs in various types of cancer has been reported to range from 0% to 29% (Soussi et al 2000, review). Such variances can be due to different tumour types, or different techniques employed in p53 AAb detection. The ELISA assay is a more sensitive method compared to immunoblotting techniques. Variations in the sample numbers analysed in each study can also contribute to the significant ranges noted.

Patients with known breast cancer and a family history (fhx) of the disease have a lower incidence of serum p53 AAbs compared to women with sporadic breast cancers (9.1% in the fhx group compared to 29.4% in sporadic group) (Green et al, 1994). This may therefore suggest that familial tumours expressing p53 antigens may be less immunogenic than those from patients who do not have a fhx of breast cancers. p53 AAbs were also detected in 11% of women without breast cancer but with a positive fhx (Green et al, 1994) thereby suggesting a likelihood of developing breast cancer in the future. This can be used as the basis of screening.

One purpose of the current study was to analyse serum samples of a large cohort of women with a positive fhx of breast cancer in order to detect the presence of one or more AAbs including to p53 as serum tumour markers. Detection of these AAbs in this specific group of women could lead to the development of a panel of various serum AAb tumour marker assays used in screening.

### 3.3. C-MYC

c-myc is an oncoprotein believed to be involved in both cellular proliferation and apoptosis.

#### 3.3.1. C-MYC STRUCTURE AND ORIGIN

The c-myc oncogene is located on the far end of the long arm of chromosome 8 (8q24). The protein product of c-myc comprises a 439 amino acid sequence, which is shown to be a 62kD phosphoprotein (Ramsay et al, 1984). It is believed to be involved in both cellular proliferation and apoptosis. Its mechanism of action is not known although it is suggested

that it binds with other proteins and induces both up and down regulation of cell cycle regulatory proteins (Guo et al, 2000).

### 3.3.2. C-MYC IN CARCINOGENESIS

In tumourigenesis the c-myc oncogene can be either amplified or over-expressed. Over-expression of the gene is not thought to be due to an increased gene copy number (Bieche et al, 1999). Inappropriate expression of c-myc can result in cellular proliferation which can then induce tumour formation. This was demonstrated more than 20 years ago when the c-myc oncogene was proven to induce tumours of the mammary gland in transgenic mice (Stewart et al, 1984). However this ability to induce tumour on its own is actually low and requires other genetic modifications such as bcl-2 expression, HER2 oncogene amplification and the inactivation of the p53 gene (Aulmann et al, 2002). Interestingly amplification of both c-myc and HER2 is rarely seen together in the same tumour, in either female (Oshima et al, 1995) or male breast tumours (Mourao-Netto et al, 2001).

The reported frequency of c-myc amplification is variable and ranges from 4% to 50% (Nass and Dickson, 1997) whilst data on gene overexpression shows prevalence in 22% of breast cancers (Bieche et al, 1999).

Work done on canine mammary tumours has shown that c-myc oncoproteins are nuclear proteins, which can stain both epithelial and myoepithelial cells (Inoue and Shiramizu, 1999). Expression of this oncoprotein in tissue samples can be detected by IHC using antibodies to c-myc such as 9E10 (Oncogene Science Inc. Manhasset, NY, USA). This enables detection of over expressed gene products. Differential polymerase chain reaction (dPCR) can be used



to measure c-myc gene amplification (Naidu et al, 2002). There appears to be differential expression of this oncogene in different breast tumour types with ductal invasive showing the highest expression and colloidal tumours the lowest (Naidu et al, 2002),

Since within the same tumour there is higher c-myc expression in invasive component compared to non-invasive part (Naidu et al, 2002), some authors (Watson et al, 1993; Robanus-Maandag et al, 2003) conclude that over expression and amplification may play an important and early role in the progression of non-invasive to invasive tumours.

### 3.3.3. C-MYC AND PROGNOSIS

Unlike MUC1 and p53, most studies indicate that the presence of c-myc either as an over expressed oncoprotein or as an amplified gene correlate with poorer prognosis (Table 3.3.). The recent meta-analysis from Deming also highlights this possible role of c-myc gene amplification in determining poorer prognosis breast cancer (Deming et al, 2000).

Table 3.3. Literature on c-myc and its correlation with breast cancer prognosis

Study	Detection method	Tissue antigen or gene amplification detected	Prognosis
Figueiredo 2007	Genotyping	Gene amplification	No correlation
Rodriguez-Pinilla 2007	ISH	Gene amplification	No correlation
Mourão Netto 2001	IHC	Tissue antigen	No correlation
Shanmugham 2004	IHC	Tissue antigen	Poor
Park 2005	FISH	Gene amplification	Poor
Schlotter 2003	PCR	Gene amplification	Poor
Naido 2002	IHC + PCR	Tissue antigen and Gene amplification	Poor
Deming 2000	Meta-analysis	Gene amplification	Poor
Scorilas 1999	IHC + PCR	Tissue antigen and Gene amplification	Poor
Lê 1999	Northern Blot	Gene amplification	Poor
Bland 1995	IHC	Tissue antigen	Poor
Pietiläinen 1995	IHC	Tissue antigen	Poor
Watson 1993	Southern Blot + PCR	Gene amplification	Poor
Borg 1992	PCR	Gene amplification	Poor

Several studies have shown oncogene amplification to be related to early recurrence, lymph node metastasis and poorer overall survival (OS) (Borg et al, 1992) (Scorilas et al, 1999) (Lê et al, 1999). This link with prognosis is due to its association with larger tumours with higher proliferative activity (Kreipe et al, 1993). However in another study there appears to be no such correlation between c-myc oncogene amplification and breast tumour characteristics (Rodriguez-Pinilla et al, 2007).

In studies that detected only the presence of the c-myc oncoprotein in invasive breast cancer, some reported a poorer outcome (Table 3.3.). Further data from Aulmann also highlighted the correlation between c-myc oncoprotein in tissue and higher grade and more aggressive subtype of non-invasive breast tumour (Aulmann et al, 2002). Mourao Netto in his study did not however show any correlation between c-myc oncoprotein found in tissue and breast cancer prognosis (Mourao Netto et al, 2001). These differences are probably due to the wide variations of detection methods used as well as whether the antigen or the gene itself was detected as demonstrated in Table 3.3.

It has also been suggested that c-myc gene expression may underlie resistance to antioestrogens such as tamoxifen in human breast cancer cells (Venditti et al, 2002; McNeil et al; 2006). Although the exact mechanisms are not fully understood, there appears to be a positive correlation between c-myc gene amplification and ER negativity. c-myc marker status may therefore be potentially used as a predictive marker of anti-oestrogen therapy.

#### 3.3.4. SERUM C-MYC ONCOPROTEIN

Although c-myc oncogene is known to express two major oncoproteins: c-myc1 and c-myc2 (Batsche and Cremisi, 1999), there is very little available data on measurement of these or any other c-myc serum oncoproteins as tumour markers. A study by Yamamoto failed to detect the presence of c-myc oncoproteins in the serum of lung cancer patients who have raised AAbs to the oncoprotein (Yamamoto et al, 1999).

#### 3.3.5. AUTOANTIBODIES TO C-MYC

AAbs against c-myc were detected in human sera by western blotting in the late nineteen eighties (Ben-Hahrez et al, 1988). Ben-Hahrez and colleagues reported that 56% (25/44) patients with colorectal cancer expressed c-myc AAbs which were significantly higher than the 17% of normal controls.

The presence of anti-c-myc antibodies has also been reported in healthy volunteers and patients with known rheumatic and autoimmune diseases such as SLE (Deguchi et al, 1989). The significance of raised c-myc AAbs in autoimmune disease is not understood, as it does not appear to correlate with disease progression or flare up (Deguchi et al, 1988). It had been suggested that some autoimmune diseases (e.g. SLE) are associated with an increased incidence of cancer (Bernatsky et al, 2005) although this is disputed (Sultan et al, 2000). The raised levels of AAbs to c-myc in this group may therefore indicate a group with raised risk of developing cancer in the future.

c-myc AAbs have been studied in various solid tumours such as breast and lung cancer. Thirteen percent of lung cancer patients (9/68) were shown to have AAbs to c-myc although the AAb was also detected in 3.3% of healthy volunteers (1/30) (Yamamoto et al, 1999). In an earlier study by the same group 10% (7/70) of lung cancer patients were noted to have L-myc AAbs (Yamamoto et al, 1996). In patients with breast cancer, c-myc AAbs were detected in both invasive and pre-invasive tumours. The overall frequency in the latter group was 13% (Chapman et al 2007). In this study the AAbs were noted only in intermediate and high-grade pre-invasive and invasive tumours. No AAbs were detected in the low-grade tumours. There were no data on survival. Similar results were also noted by Megliorino (12.3%) (Megliorino et al, 2005) and Zhang (18.8%) (Zhang et al, 2003). Again, prognostic significance of c-myc was not detailed in either study.

c-myc AAbs have also been detected in the serum of patients with African Burkitts lymphoma as well as other forms of blood borne malignancies (LaFond et al, 1992). In the former malignancy, translocation of a chromosome results in altered c-myc expression thereby causing the disease (Kelly and Rickinson, 2007). However raised levels of c-myc antibodies are not necessarily associated with conditions that have increased expression of c-myc (LaFond et al, 1992) and significance with regards to tumour prognosis and therapy is not well established.

## Chapter 4

### Hypothesis, Aims and Overview

#### 4.1. HYPOTHESIS OF CURRENT THESIS

We hypothesised that an immune response to various tumour-associated antigens is an early event in breast cancer evolution and the detection of these AAbs can lead to earlier breast cancer diagnosis. Detecting AAbs are more appropriate than their respective antigens as AAbs are the amplified signal of the antigens and so are more readily detectable in early cancer evolution.

#### 4.2. AIMS OF CURRENT THESIS

This thesis is a continuation of previous work at the Nottingham Breast Unit that had developed a new panel of assays for the detection of AAbs to MUC1, p53, c-myc and HER2 in breast cancer (Cheung 2001).

Based on the above hypothesis we aimed to detect AAbs as serum tumour markers to a panel of three specific antigens in several breast cancer populations using an updated version of the in-house prototype ELISA assay. The populations studied had a longer follow up compared to earlier data. They included those at-risk in terms of recent high-risk histology or those with known high-risk flx. The panel of markers were detected in this group to establish its role in screening.

Women with primary breast cancer were also studied to establish the role of the panel of markers in diagnosis and prognosis. The three serum tumour markers chosen represented various mechanisms and steps during the process of tumourigenesis and included AAbs to MUC1, p53 and c-myc.

### 4.3. OVERVIEW OF CURRENT THESIS

Our work endeavoured to develop and utilise assays to detect tumour marker AAbs in the serum of various populations:

- Detection of one or more AAbs in women with no known risk or disease i.e. the Normal population.
- Detection in those with benign breast disease.
- Detection of one or more AAbs in those with known raised fhx risk.
- Detection in those with histological at-risk (ADH).
- Detection in those with pre-invasive breast cancer.
- Detection in those with screen detected breast cancer.
- Detection in those with symptomatic breast cancer.

By accurately measuring serum AAbs as tumour markers in these populations we aimed to:

- Establish appropriate cut-off values.
- Establish stability of signal over prolonged time.
- Attain sensitivity and specificity values of serum AAbs to p53, MUC1 and c-myc as tumour markers either as a single marker or in combination in the 'at-risk population'.
- Be able to use the markers singly or combined as a diagnostic tool in those with PBC.
- To be able to predict tumour parameter by pattern of serum AAb marker detection in those with proven PBC.
- To be able to prognosticate PBC patients depending on the pattern of AAb detection.



## Chapter 5

### Methods and Materials: Samples

## 5.1. INTRODUCTION

The local ethics committee approved the study and patients participating in the study were given written information leaflets prior to clinic appointment giving details of the study, sample storage and the levels of participation requested. Opportunities to ask questions about the study were given. All patients in the study gave written informed consent. Copies of the information sheet and the signed consent forms were stored for record purposes.

The author saw patients during the study period in either the outpatient family history clinic when the patients were examined and investigated with a mammogram as part of their routine clinical management, or at pre-assessment clinic prior to their surgery. Blood samples from fhx, DCIS, screen-detected and primary breast cancer patients were collected by the author over a 2-year period from January 2002 to December 2003. The author attended several of the above clinics per week to collect the samples personally. Sample collection was by standard venepuncture technique under antiseptic conditions. This study has extended over a number of years and previous Research Fellows had collected some of the blood samples used in the study. These samples dated back as much as 12 years and were stored in freezers at -20 °C. Some of these samples were from all the above populations and therefore samples used in the study had a mixture of those collected by the author and some collected by previous Research Fellows. All collected blood samples were allowed to clot at room temperature and centrifuged at 1000G for 20 minutes. The sera were then aliquoted into labelled tubes and frozen in 1ml aliquots at -20°C prior to use.

## 5.2. TUMOUR PROGNOSIS

In the study, the role of AAbs to MUC1, p53 and c-myc oncoproteins as serum tumour markers in prognosis was elicited. Prognosis was established by two separate means. We compared tumour specific parameters such as tumour size, grade and lymph node status to the presence of the AAbs. We also attained follow up data on PBC patients to establish recurrence (local, regional and distant) and overall survival (OS). Presence of AAb in the serum of patients who donated samples and recurrence and OS were compared to attain prognostic value of the markers.

Tumour specifics were attained from hospital breast pathology reports whilst data on recurrence, OS and follow up from the unit's up-to-date database and patients notes where relevant.

The parameters noted were:

- Tumour size in cm
- Tumour grade
- LN status
- VI
- ER status
- NPI

### 5.3. POPULATIONS STUDIED (Table 7.1.)

Serum samples from various populations were analysed in this project.

#### 5.3.1. CONTROL GROUP

The control group was the combination of both Normal and Benign populations. This group was the non-malignant, not 'at-risk' group. Normal and Benign groups were combined to enable a larger population to be used as a control thereby increasing the accuracy of the cut-off values. The two groups were similar in age and sex of patient. They differed in sample age, as the Benign samples were stored significantly longer than the Normal samples (Table 1). Comparison between the mean optical density (OD) of older and more recent samples for both groups were made to determine whether there were any differences. Significant differences would compromise combining the two mis-matched groups in terms of age of samples to elicit an accurate cut-off value.

All control group samples were analysed together initially to attain two arbitrary cut-off values (set at Mean OD signal (650nm) + 2 or + 3 Standard Deviations). Against these values studied populations were qualitatively denoted 'positive' or 'negative' for the analysed AAb.

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##### 5.3.1.1. NORMAL

Samples of blood from patients who had no known breast diseases were analysed. Well women attending the National Health Service Breast Screening Programme (NHSBSP) (aged

50 – 64) or the Age Trial (aged 40 – 50) were consented to donate blood for the study as Normal controls in two separate occasions; mid 1996 and mid 2000 by the previous Research Fellow (Cheung 2001). Twenty-eight samples were collected in the former period whilst 16 in the latter. Full ethical approval was attained for this aspect of the study. The women were proven on mammogram to be negative for breast cancer and had no family history of the disease. In total, only 44 samples were available for study.

#### 5.3.1.2. BENIGN

Benign breast disease is the most common presentation to the breast clinic (Thrush et al, 2002). This group presented with either benign symptoms e.g. mastalgia, nipple soreness or nipple discharge or with histological benign breast disease e.g. fibroadenoma, papilloma and benign breast changes. Malignant breast pathology was excluded using clinical and imaging techniques. Histology showing cellular hyperplasia was accepted as benign changes but those with cellular atypia were excluded from this group.

Benign group patients were consented to donate blood samples by previous Fellows at the benign breast clinic in two separate occasions. Thirty-four samples were from women who were diagnosed in 1987 of benign symptoms or histology. The remaining samples were collected by KL Cheung in 1999 for his study (personal communication). The exact numbers of cases in each benign category was not known although the case notes of three patients who were positive for one or more AAb markers were subsequently reviewed. By the end of author's tenure at the unit (March 2004), none of the benign groups had proceeded to breast cancer.

### 5.3.2. AT-RISK GROUP

#### 5.3.2.1. FAMILY HISTORY

All women attending the fhx clinic at Nottingham Breast Unit from January 2002 to December 2003 were requested by the author to donate a 20ml sample of blood. Patients were consented to this and entry into the study did not alter patient management. Each patient was categorised into low, moderate and high risk of developing breast cancer depending on degree of family history of breast and/or ovarian cancer.

Hospital protocol for defining category of risk at the time of writing the thesis was the following:

- Low risk – Single member of first-degree family with breast cancer diagnosed at any age above 50 years. Despite the overall low risk of developing breast cancer in this group, the risk was still higher than the normal population (Collaborative Group on Hormonal Factors in Breast Cancer, 2001).
- Moderate risk – Single member of first-degree family with breast cancer diagnosed at age less than 40 years. This translated into an absolute risk of developing breast cancer by 50 years of 5% and lifetime risk of 17%.
- High risk – Two or more members of first-degree family with breast cancer diagnosed at age less than 40 years. The presence of ovarian or bilateral breast cancers increased the risk further. This translated into an absolute risk of developing breast cancer by 50 years of 8% and lifetime risk of 30%.

Concurrent breast pathology was ruled out by standard clinical and imaging investigations (e.g. mammography and ultrasound) as per hospital protocol. Patients were requested to give repeat samples when they returned to clinic which was initially 18 month follow up irrespective of risk but later changed to yearly follow up for those stratified into the high-risk group. Therefore some patients were seen only once during the study period whilst others were seen twice. Standard venepuncture technique was carried out in attaining blood samples, which were collected in 2 EDTA bottles and 2 normal 4ml bottles. By the end of the author's tenure at the unit, thorough review of the database had confirmed 13 patients had progressed to breast cancer from this group. Case notes of this group were analysed for follow up data and cancer specifics.

#### 5.3.2.2. BRCA 1 OR 2 POSITIVE FAMILY HISTORY

Patients who were known to have a mutation in the BRCA 1 or 2 genes were also enlisted into the study by the author. Donated blood was collected during routine fhx clinic and stored and analysed as previously. Standard venepuncture technique was used in the collection of samples. Concurrent breast pathology was excluded on the basis of a normal clinical and radiological examination. No patients were known to have progressed to breast cancer by end of study period.

#### 5.3.2.3. HISTOLOGICAL AT-RISK

Women with known high-risk pathology i.e. ADH were consented to donate 20ml of blood by the author. These women normally attended the family history clinic where consent and samples were attained. None progressed to cancer at end of study period.

### 5.3.3. SEQUENTIAL SAMPLES

Some women in the fhx group had already consented to give blood samples to previous Research Fellows as part of the ongoing study and so sequential samples were available from some of these fhx cases. Some women on the ATAC trial (Appendix) consented to donate blood samples for the study. The first sample was at pre-op assessment and the sequential samples were donated at six monthly interval after surgical treatment and whilst on the trial. All patients on the trial were on an anti-oestrogen, though the exact medication was blinded from the author.

### 5.3.4. PRIMARY BREAST CANCER (PBC) GROUP

#### 5.3.4.1. PRE-INVASIVE BREAST CANCER

Women recently diagnosed with DCIS were consented by the author to donate 20ml of pre-operative blood for the study. They attended the day-case pre-clerking where consent for entry into the study was attained.

#### 5.3.4.2. SCREEN-DETECTED BREAST CANCER

Those recently diagnosed with a screen detected breast cancer were consented by the author at day-case pre-clerking to donate 20ml of pre-operative blood for the study.



#### 5.3.4.3. SYMPTOMATIC BREAST CANCER

All women diagnosed with symptomatic breast cancer, attending pre-clerking were requested by the author to donate 20ml of pre-operative blood.

#### 5.3.5. SERUM SAMPLES FROM OUTSIDE THE STUDY PERIOD

Cryoprecipitate serum samples from 1987 onwards were also used in the study. They included patients with:

- No known breast cancer or disease (NORMALS)
- Benign breast disease
- Pre-malignant breast disease (ADH)
- Primary breast cancer

Previous clinical fellows had obtained consent and collected these samples.

## Chapter 6

### Method and Materials: Research Methods

## 6.1. VALIDATING THE ASSAY

To validate the assay and the results attained, impact of sample age, reproducibility and reliability of assay were established.

### 6.1.1. SAMPLE AGE AND SIGNAL STRENGTH

Since all our samples were donated over a large period of time and AAb signal may fade over time we established whether sample age would impact the results. We compared the median OD (650nm) values of two large cohorts of PBC samples taken at two separate periods separated by a decade.

### 6.1.2. REPRODUCIBILITY OF ASSAY

#### 6.1.2.1. INTER-ASSAY REPRODUCIBILITY

Inter-assay reproducibility of the lab technique was done by analysing same sample on 3 different occasions and establishing the coefficient of reproducibility (CR) of the samples. This was termed as the Bland Altman CR (Bland and Altman, 1986). In practice the mean OD (650nm) of 20 fhx, 20 PBC and 20 ABC samples were analysed on three separate occasions. The results of the repeated samples were calculated:

The formula used was: 
$$CR = 1.96 \times \sqrt{(\Sigma(d2-d1)^2 / (n-1))}$$

This indicates that 95% of the time, two or more readings of the same sample at different times will not be any further apart than the CR value for the sample.

#### 6.1.2.2. INTRA-ASSAY REPRODUCIBILITY

Intra-assay variability by each sample was calculated by measuring the samples as triplicates for both the antigen and its negative vector only lysate (VOL) control. The coefficient of variability (CV) was measured for each antigen and control.

CV was measured by the following equation:

$$CV = (\text{standard deviation (SD) of triplicates} / \text{Mean of triplicates}) \times 100$$

This indicates the variation as a percentage, which may occur about the mean of the triplicates (Holme, 1983). Triplicates with CVs greater than 10 were repeated until an acceptable CV was attained (i.e.  $CV < 10$ ). The mean sample (antigen or VOL) value was therefore always within a CV less than 10% as anything greater than this value was repeated until the variation in the triplicate wells were acceptable.

#### 6.1.3. RELIABILITY

To assess the reliability of the assay to detect AAbs to the panel of antigens in the various populations, we needed to establish whether the assay was able to distinguish between cancer and non-cancer control group. We therefore compared AAb detection in the control population to patients with known breast cancer i.e. PBC group. Significantly higher detection rate in the PBC group compared to the control population would indicate reliability of the assay to detect AAbs to the tumour-associated antigens.

## 6.2. ENZYME LINKED IMMUNOSORBANT ASSAY

ELISA is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. It uses two antibodies. For the purpose of our study the first antibody is the AAb to be determined in the sample. This antibody is specific to the antigen i.e. MUC1, p53 and c-myc which is coated onto microtiter wells. The second antibody is added to the wells in order for it to react to any antigen-antibody complexes formed. This antibody is coupled to an enzyme. The enzyme allows detection of the antibody through a fluorogenic reaction with a substrate, which is measured by a photospectrometer.

Our ELISA gave a qualitative result, which just gave a 'positive' or 'negative' result dependent on a determined cut-off value. No quantitative information was gained from the strength of the signal. Determining the exact cut-off is described in detail in later chapters.

### 6.2.1. ANTIGEN PRODUCTION: MUC1, P53 AND C-MYC

All antigens were provided for this study by Nottingham University Tumour Immunology Group. They were MUC1, p53 and c-myc. The following is a brief overview of production of these antigens used by the author.

The recombinant antigens were produced according to in-house protocols. Specific cDNAs encoding the genes p53 and c-myc were amplified by PCR and then cloned into the pET21b expression vector (Novagen, Darmstadt, Germany). The cDNAs were tagged with a histidine tag in the N terminal that allowed the purification of the resulting protein and a BirA tag in the C terminal. The latter tag allowed biotinylation of the resultant protein.

Plasmids incorporating the above-tagged cDNAs were then transformed into E.coli BL21 (DE3) bacteria (Novagen) where the recombinant proteins were expressed. The bacteria were cultured in CYM media.

The bacterially expressed recombinant proteins were then purified on His-Trap nickel affinity chromatography columns (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol and refolded by dialysis. Specificity and purity of these proteins were confirmed by SDS PAGE. A negative control protein (i.e. expressed vector with dual tags alone without the cDNAs for either p53 or c-myc) was also produced under identical conditions. This was termed as the Vector Only Lysate or VOL and was used as a control.

Affinity purified human MUC1 antigen was also provided by the Tumour Immunology Group and produced as described by O'Sullivan et al 1990 (O'Sullivan et al, 1990). The MUC1 used here was from a pleural effusion from a single patient with advanced breast cancer.

## 6.2.2. ELISA TECHNIQUE AND CHANGES TO PREVIOUS PROTOCOL

Single batches of antigens (MUC1, p53 and c-myc) were used in the autoantibody assays. The ELISA assays for each of the above antigens were modified from the original protocol. Earlier data had shown a high background signal that potentially could mask the true signal from detected AAbs. The alterations to the assays for the three markers attempted to reduce non-specific binding and hence high background signal. The following is a brief account of the original protocol from our unit (Cheung 2001) as well the final version of the protocol as used by the author. The changes in the two assays have been highlighted.

### 6.2.2.1. MUC1

#### 6.2.2.1.1. ORIGINAL PROTOCOL FOR MUC1 (CHEUNG 2001)

MUC1 was isolated from a pool of serum of 20 women with known ABC. 50 $\mu$ l of antigen diluted 1/10 in PBS was air-dried overnight. The plate was washed once with Tween to remove residual salt crystals. It was then blocked to reduce non-specific binding with 2% PVP in PBS and incubated for one hour. The plate was washed again 3 times with Tween. The diluted serum was plated (50 $\mu$ l per well) in triplicate. It was further incubated for one hour and then washed 4 times with PBS/Tween.

50  $\mu$ l of conjugated anti-species Mab labelled with HRP (Dako) were added to each well and incubated for one hour. After washing 4 times with PBS/Tween TMB was added (50 $\mu$ l/ well) and the plate read kinetically over a 10-minute period at A650 nm. Readings ( $V_{max}$  = rate of reaction (maximum velocity) in milli-optical density per minute (mOD/min). An intra-assay CV <10% was accepted.

#### 6.2.2.1.2. CURRENT PROTOCOL FOR MUC1

MUC1 antigen was derived from the pleural effusion of a single ABC patient. Its concentration was not known although it was verified against previous batches by direct analysis with serum CA15.3 (personal communication). It was diluted 1:10 in PBS. 50µl of the antigen solution was aliquoted into each well of a micro-titre plate and dried down at room temperature over night.

The plate was washed four times with PBS + 0.1% Tween (2L 5 X PBS + 8L dH<sub>2</sub>O) using 250µl of the solution per well and then blocked with 100µl of 0.1% casein per well (1g casein in 100ml PBS + 0.1% Tween). The plate was incubated and shaken at room temperature for 1 hour and then further washed x4 with PBS + 0.1% Tween using 250µl per well.

The human serum was diluted 1:100 with high salt PBS solution and pre-incubated on rollers for 2 hours prior to use. After washing of the plate, 50µl of the diluted serum sample to be tested was added to each well (3 wells per sample) and incubated at room temperature with shaking for 1 hour. After incubating the sample to be tested with known MUC1 antigen in the wells the plate was again washed x4 with PBS + 0.1% Tween using 250µl per well.

50µl of a secondary antibody i.e. peroxidase conjugated rabbit anti human (Dako cytometry, Cambridgeshire, UK) was added to each well and incubated at room temperature with shaking for 1 hour. The plate was washed x4 with PBS/Tween using 250µl per well.

In the final stages of the protocol 50µl of TMB substrate (1 TMB/DMSO aliquot (75µl) + 1.3µl 30% H<sub>2</sub>O<sub>2</sub> + 10ml Sodium Acetate buffer) was added to each well and read 10 minutes



later at end-point OD at 650nm using an Asys Expert plate reader and MikroWin 2000 software. A mean of the triplicate values for each sample was designated the value for the sample. A sample who's CV of the triplicates was greater than 10 was ignored and repeated again.

#### 6.2.2.1.3. CHANGES FROM ORIGINAL PROTOCOL

MUC1 in current protocol was isolated from the pleural effusion of a single patient rather than from a pool of sera from patients with ABC. There was a greater number of washing with PBS/Tween in our assay compared to original assay. This was to remove more residual salts, which may result in higher background signal.

In the current protocol the plates were blocked with casein in PBS rather than PVP in PBS. PVP in high concentrations is known to lead to protein aggregation and precipitation (Gombotz 1994) and result in decreased binding of the rabbit antihuman antibody onto the wells. Casein is a well-established blocking agent and has been reported to block up to 90% of non-specific background signal (Vogt 1987) and therefore was used in preference to PVP in our study. The human serum sample was pre-incubated in high salt PBS for 2 hours prior to use. In-house data noted a reduction in background signal in samples that were diluted and pre-incubated for two hours (data unpublished).

In our protocol the plates were read as an end-point after ten minutes rather than the kinetic reading over this 10-minute period. This gave an easier end-point for qualitative results.

#### 6.2.2.2. P53 / C-MYC

##### 6.2.2.2.1. ORIGINAL PROTOCOL FOR P53 / C-MYC (CHEUNG 2001)

50µl of 1 µg/ml of avidin was pipetted into each well and the plate air-dried over-night. The plate was washed with Tween, blocked with PVP in PBS and then re-washed 3 times. Biotinylated antigen (diluted 1/10 in PBS) was plated out at 50µl per well. The plate was further incubated for one hour and then washed 4 times with PBS/Tween.

The serum was diluted 1/100 in PBS and pipetted in triplicate (50µl per well). Appropriate commercial Mab were used as +ve and -ve controls. After incubation and washing 4 times, conjugated anti-species Mab labelled with HRP were added to each well (50µl per well).

In the final step of the assay 50µl TMB was added per well after the plate was incubated for one hour and washed. The plate was read kinetically over a 10-minute period at 650nm. Readings ( $V_{max}$  = rate of reaction (maximum velocity) in milli optical density per minute (mOD/min) with an intra-assay CV <10% were accepted.

##### 6.2.2.2.2. CURRENT PROTOCOL FOR P53 / C-MYC

Wells were coated with 50µl neutravidin at 2.5µg/ml in PBS. The plate was incubated and covered in parafilm over night at 4°. The plate was washed with PBS + 0.1% Tween four times and air-dried.

Both the recombinant antigens and VOL were diluted to 0.5µg/ml in 0.1% casein/0.05% Tween 20/0.5M NaCl/PBS. 50µl of the diluted antigen was then added to wells number 1, 2, 3, 7, 8 and 9 of each row. Equal volume and concentration of diluted VOL was added to the intervening wells i.e. numbers 4, 5, 6, 10, 11 and 12 of each row. The plate was further incubated at room temperature, whilst shaken and covered, for 30 minutes. The plate was again washed with PBS + 0.1% Tween 4 times.

Human serum samples diluted to 1:100 in 0.1% casein/0.05% Tween 20/0.5M NaCl/PBS were pre-incubated and shaken on rollers for 2 hours. 50µl of the diluted human serum sample to be tested was then added into both the antigen and VOL wells. This was then further incubated whilst covered and shaken at room temperature for another 2 hours.

After incubation the plate was washed 4 times with PBS + 0.1% Tween. 50µl of a secondary antibody i.e. peroxidase conjugated rabbit anti human antibody (Dako cytometry, Cambridgeshire, UK) was added to each well (antigen and VOL wells) and incubated at room temperature whilst shaken for 1 hour. As before, the plate was washed x4 with PBS + 0.1% Tween.

In the final stages of the protocol 50µl of TMB substrate (1 TMB/DMSO aliquot (75µl) + 1.3µl 30% H<sub>2</sub>O<sub>2</sub> + 10ml Sodium Acetate buffer) was added to each well and allowed to develop for 10 minutes. The plate was then read 10 minutes later at end-point OD at 650nm using an Asys Expert plate reader and MikroWin 2000 software. A mean of the triplicate values for each sample was designated the value for the sample.

#### 6.2.2.2.3. CHANGES FROM ORIGINAL PROTOCOL

A reliable and stable recombinant HER2 antigen was not available for this project.

Neutravidin was air-dried onto plates in preference to avidin. Neutravidin is a modified avidin that provides biotin-binding characteristics of avidin but with reduced non-specific binding. Avidin is heavily glycosylated, which results in non-specific binding. In contrast, neutravidin has no carbohydrate and therefore reduced non-specific binding (Hiller 1987).

There were more washing with PBS/Tween in the current protocol. This increased the removal of salt residue, which would otherwise result in higher non-specific binding.

Blocking the coated wells with PVP has been abandoned since PVP in high concentrations results in protein aggregation and precipitation (Gombotz 1994). It also decreases binding of the rabbit antihuman antibody onto the wells. Casein was used in preference as a blocking agent due to its high blocking characteristics (Vogt 1987).

In the current protocol the human serum was pre-incubated for 2 hours in order to reduce background signal due to non-specific binding (data unpublished).

VOL was used to control for background and non antigen-specific binding (e.g. anti-biotin) for both p53 and c-myc assays in the current protocol. A high background signal was still noted on initial data despite attempting to minimise this with the above alterations in protocol. The high background signal was due to non-specific binding of antibodies to both to the vector only lysate as well as other non antigen-specific sites in the well. The vector signal

was subtracted from the antigen signal (which also contains vector proteins). This VOL corrected signal was deemed to be the true signal for the sample.

Cut-off value for both p53 and c-myc positivity was generated according to the following protocol: the VOL corrected signal was analysed for each sample of the control population. The cut-off was calculated as the mean VOL corrected signal of all control sera + 2 or 3 standard deviations (SD) of the mean (Section 6.3). This is in contrast to original protocol where no correction for non-specific binding was applied. In our protocol the plates were read as an end-point after ten minutes rather than the kinetic reading over this 10-minute period. This gave an easier end-point for qualitative results.

### 6.3. ATTAINING CUT-OFF VALUES FOR POSITIVE AND NEGATIVE RESULTS

This was a qualitative study. Cut-off values were arbitrarily chosen at two different levels as described later in this thesis (Section 7.4). Any signal above these levels was designated positive whilst any signal below was negative. No quantitative information was gained from the actual signal strength.

Cut-off values were attained from the control population, which consisted of both Normal (section 5.3.1.1) and Benign groups (see section 5.3.1.2). Neither of the above populations was at-risk or had developed breast cancer by the end of the author's tenure at the unit (Mar 2004).

In total, 44 Normals and 66 Benign cases were combined to provide the Control population. The two populations had similar age and sex distribution. Due to differences in their sample

storage age profiles, comparison between median signal strength of older and recent samples was made. This was in order to determine that there was no reduction in signal over the storage period before the groups were combined.

The 110 serum samples found were analysed using the above protocols to determine the cut-off values for MUC1, p53 and c-myc. Two separate cut-off values were calculated i.e. Mean + 2SD and Mean + 3SD.

The two different values enabled detection of AAbs for the various populations for specific conditions i.e. in assessing at-risk samples for screening the higher cut-off value (Mean + 3SD) would be more appropriate as this will reduce false positives (increase specificity). Screening tools need a high specificity, as false positives in screening large groups of patients would over-burden any screening programme. When the assay is used to aid diagnosis in symptomatic cases the sensitivity of the diagnostic tool is also important. To increase sensitivity a lower value (Mean + 2SD) is also calculated. In the initial part of the study we analysed using both values. In the latter part only the higher value was used as earlier results suggested excessively high false positive rates with lower cut-off values.

The MUC1 AAb serum marker cut-off value was determined as the mean OD (650nm) of the 110 Control + 2SD as well as Mean + 3SD. The ELISA technique for p53 and c-myc also generated an OD value for the VOL, which is the negative protein control for the individual sample.

Determining the cut-off values for both p53 and c-myc AAb markers is detailed in section 6.2.2.2.3. In calculating the mean values of both p53 and c-myc all negative differences

between the antigen and VOL were taken as 0. This was because a negative value denoted higher signal for the VOL control compared to the antigen and therefore implied no signal from the antigen itself.

#### 6.4. STATISTICS

All data was collected in Microsoft Excel 2000 database (Microsoft software Inc) and analysed using the Prism 4 Statistical Package (GraphPad Software, USA) by the author himself. Statistical assistance was gained from Professor Sarah Lewis at University of Nottingham. Reproducibility was calculated using the Bland Altman method for calculating coefficient of reproducibility. Normality tests were performed for all populations (Table 7.2). Medians of two populations that were not Normally distributed were compared by Mann-Whitney non-parametric two-tailed test for significance. Medians of three or more of non-Gaussian populations were compared by the Kruskal-Wallis test for significance. Categories were compared by fishers exact t test. Prognosis in terms of both survival and recurrence whether local, regional and distant metastasis were compared using the Kaplan-Meier survival curve with the log rank test for significance. In all forms of statistical analysis P values less than 0.05 were regarded as statistically significant.

## Chapter 7

### Controls: Normals and Benigns



## 7.1. SAMPLES ANALYSED

Between the periods 01/01/2002 to 01/01/2004 488 serum samples from 464 patients were collected and analysed for the research. A further 410 serum samples were included into the study from previous collections and analysed by the author.

Amongst the recently collected samples 192 were from the fhx study group of which 14 were known cases of ADH. Twenty patients in the fhx group were known to have positive BRCA genes. 296 samples from the recently collected group were those with PBC of which 119 were screen detected and 66 had developed DCIS. Previously collected samples included 66 Benign, 189 fhx (11 known cases of ADH), 111 PBC and 44 Normal cases. The Normal and Benign cases were combined to attain our cut off values for each of the three tumour AAb markers measured.

Table 7.1. Total number of samples in each study group with respective demographics

	Total number	Mean age (years)	Mean sample age (months)
Control (Normal + Benign)	110	58.8 (34 – 90)	108 months (38 - 200)
Normal	44	58.2 (34-78)	66 months (38-83)
Benign	66	60.5 (40 -90)	132 months (38 –200)
At-Risk (fhx + ADH)	381	50 (24 – 100)	47 months (20 – 142)
fhx	356	47 (24-81)	41.2 months (20 – 55)
ADH	25	74.6 (50-100)	126 months (116 – 142)
PBC-overall	407	62.9 (31-94)	69.2 months (3 – 144)
DCIS	66	60 (40-87)	21.2 months (3 –86)
Screen detected	119	63 (50-83)	29.6 months (4 – 104)
Symptomatic	222	65 (31-94)	104.3 months (4 – 144)

Table 7.2. Normality testing to assess whether Gaussian distribution for each of the 3 markers

Population	MUC1		p53		c-myc	
	KS	Normality?	KS	Normality?	KS	Normality?
Control	0.2223	No	0.1977	No	0.3742	No
At-risk	0.1437	No	0.1337	No	0.1155	No
PBC	0.1601	No	0.2967	No	0.1992	No

## 7.2. CONTROL SAMPLES

One hundred and ten samples (44 Normal and 66 Benign) were used as the control to attain the cut off values for positive results of all 3 AAbs. These women were the negative controls as they were designated not to have any family history risk or malignant breast disease at the time of writing this thesis. The two groups had similar age and sex distribution (Table 7.1). Mean sample age of the two populations varied (66 months Vs 132 months). Comparison between recent and older samples in each group noted no significant difference in the mean for any of the three AAb markers in both Normal and Benign groups (Figs 7.4, 7.5 and 7.6). This confirmed that the differing sample age of the two groups did not contribute to any inherent errors. All samples were taken by previous Research Fellows but analysed by the author in October 2003.

Table 7.3. Frequency of AAbs at Mean + 2SD and Mean + 3SD cut-off values for Control

<b>Marker</b>	<b>Positive n (%)</b> <b>Mean + 2SD</b>	<b>Positive n (%)</b> <b>Mean + 3SD</b>
MUC1 AAb	5 (4.5%)	3 (2.7%)
p53 AAb	7 (6.3)	3 (2.7%)
c-myc AAb	7 (6.3%)	5 (4.5%)
Panel of Markers	16 (14.5%)	10 (9.1%)
Any 2 positive markers	3 (2.7%)	1 (0.9%)
All 3 positive markers	0 (0%)	0 (0%)

### 7.2.1. NORMAL SAMPLES

Forty-four women with no known breast disease were classified as Normals. Time period for samples taken and analysed are detailed in Section 5.3.1.1. Mean age of the group was 58.2 years of age (Range 34-78).

Table 7.4. Frequency of AAbs at both cut-off values for Normal samples

<b>Marker</b>	<b>Positive n (%)</b> <b>Mean + 2SD</b>	<b>Positive n (%)</b> <b>Mean + 3SD</b>
MUC1 AAb	4 (9.1%)	3 (6.8%)
p53 AAb	5 (11.4%)	3 (6.8%)
c-myc AAb	5 (11.4%)	4 (9.1%)
Panel of Markers	11 (25%)	9 (20%)
Any 2 positive markers	3 (6.8%)	1 (2.2%)
All 3 positive markers	0 (0%)	0 (0%)

### 7.2.2. BENIGN SAMPLES

66 samples of patients with known benign breast disease were analysed. Mean age of this group was 60.5 (range 40 -90). There were no data available on histology and its distribution although all patients were known to have benign symptoms or histology. There were no malignant cases by the end of the study period.

Table 7.5. Frequency of AAbs at both cut-off values for benign samples

<b>Marker</b>	<b>Positive n (%)</b> <b>Mean + 2SD</b>	<b>Positive n (%)</b> <b>Mean + 3SD</b>
MUC1 AAb	1 (1.5%)	0 (0%)
p53 AAb	2 (3%)	0 (0%)
c-myc AAb	2 (3%)	1 (1.5%)
Panel of Markers	5 (7.5%)	1 (1.5%)
Any 2 positive markers	0 (0%)	0 (0%)
All 3 positive markers	0 (0%)	0 (0%)

Table 7.6. Pathologies of 3 benign cases which were positive at lower cut-off value

<b>Sample number</b>	<b>Positive marker</b>	<b>Pathology</b>	<b>Smoker</b>
842	MUC1 AAb	Intraductal papilloma	Non-smoker
857	p53 AAb	Radial scar	N/A
809	p53 AAb	Fibroadenoma	Smoker

The following graphs compare distribution of the Control with Normal and Benign groups.

Fig.7.1. Distribution of Normal / Benign and Control samples for MUC1 AAb

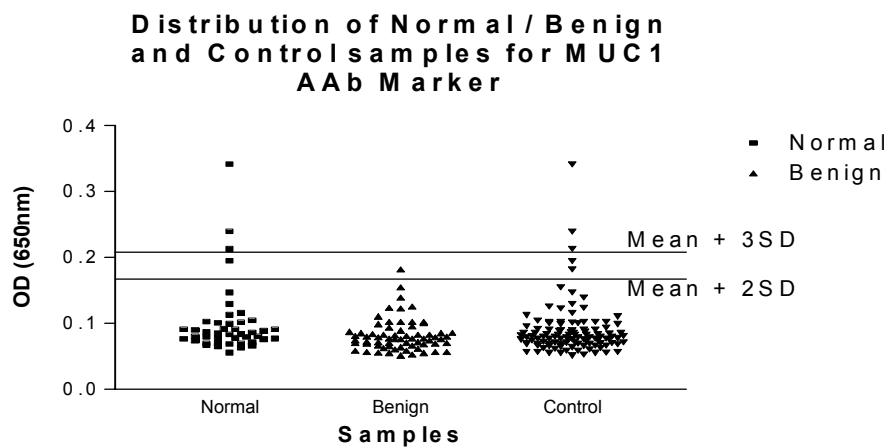


Fig.7.2. Distribution of Normal / Benign and Control samples for p53 AAb

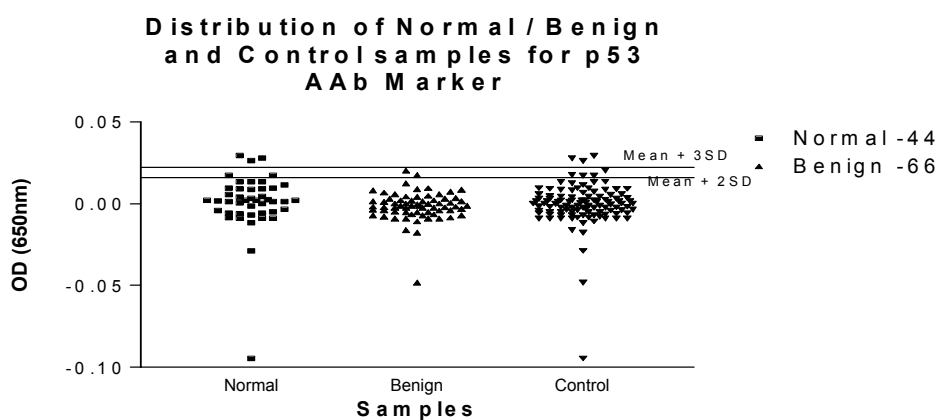
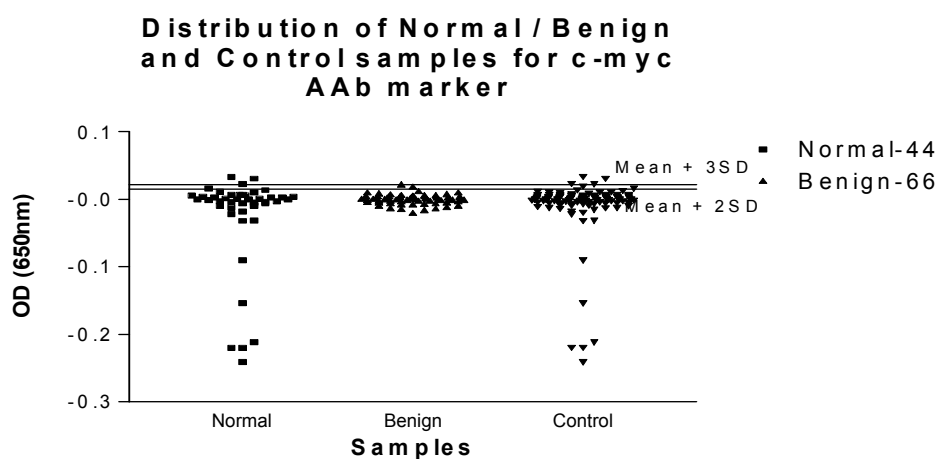


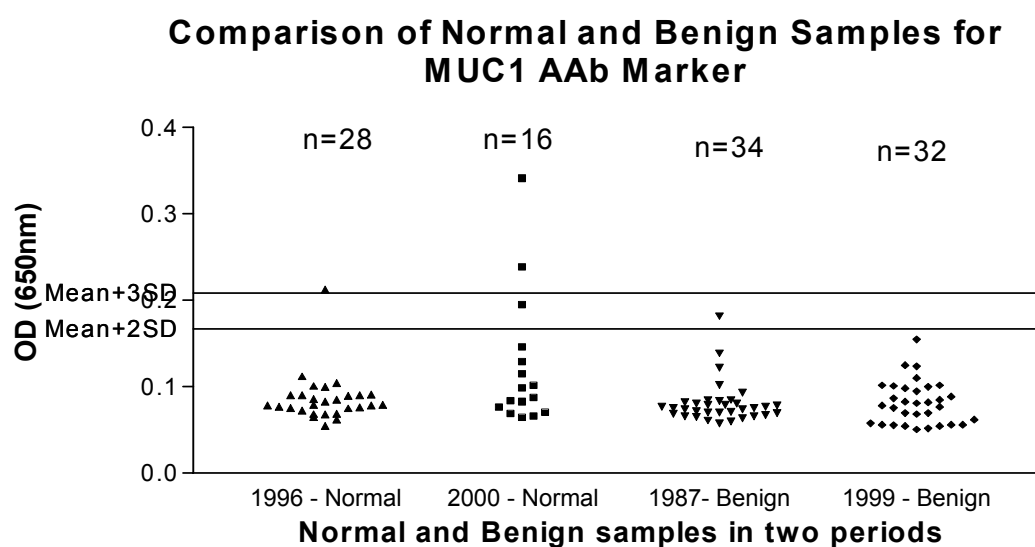
Fig.7.3. Distribution of Normal / Benign and Control samples for c-myc AAb



### 7.3. COMPARISON OF SAMPLE AGES FOR NORMAL AND BENIGN GROUPS

Both Normal and Benign samples were collected in different periods (Section 5.3.1). Signal from older samples may fade. There is marked age discrepancies between the samples. This will reduce the reliability of combining the two groups. We therefore determined to compare the signal difference between older and recent samples in both Normal and Benign populations.

Fig.7.4. Comparison of older and more recent Normal and Benign samples for MUC1 AAb



Normal:

Median OD (650nm) 1996 - 0.0795 (Range 0.0550 – 0.2127) N=28

Median OD (650nm) 2000 - 0.09333 (Range 0.06467 – 0.3413) N=16

P = 0.1304 (non-significant)

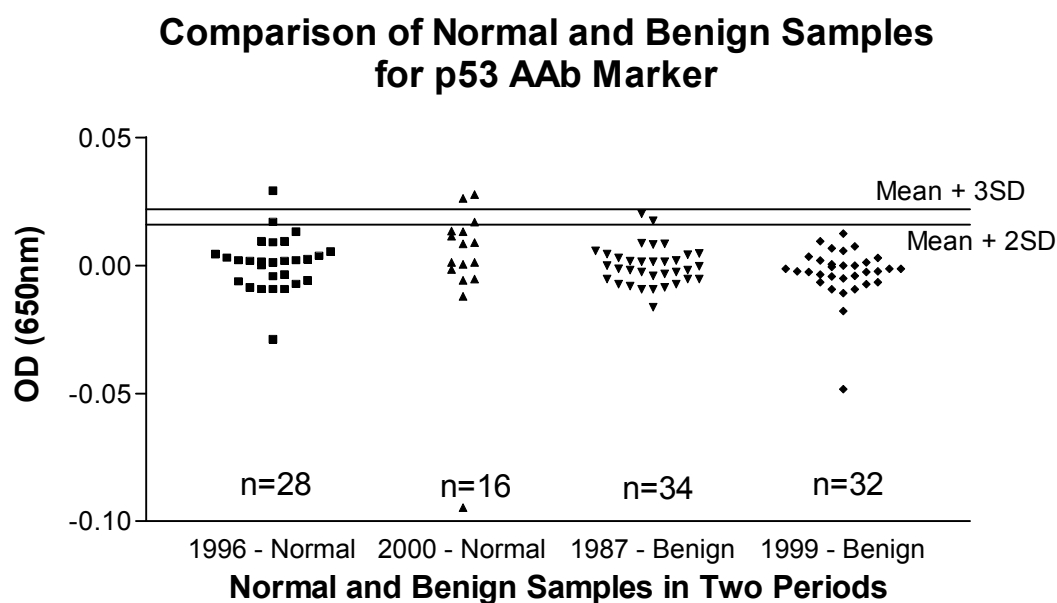
Benign:

Median OD (650nm) 1987 - 0.07583 (Range 0.0580 – 0.1817) N=34

Median OD (650nm) 1999 - 0.0800 (Range 0.05067 – 0.1547) N=32

P = 0.8575 (non significant)

Fig.7.5. Comparison of older and more recent Normal and Benign samples for p53 AAb



Normal:

Median OD (650nm) 1996 - 0.001667 (Range -0.0290 – 0.0290) N=28

Median OD (650nm) 2000 - 0.004667 (Range -0.09467- 0.02767) N=16

P = 0.3602 (non significant)

Benign:

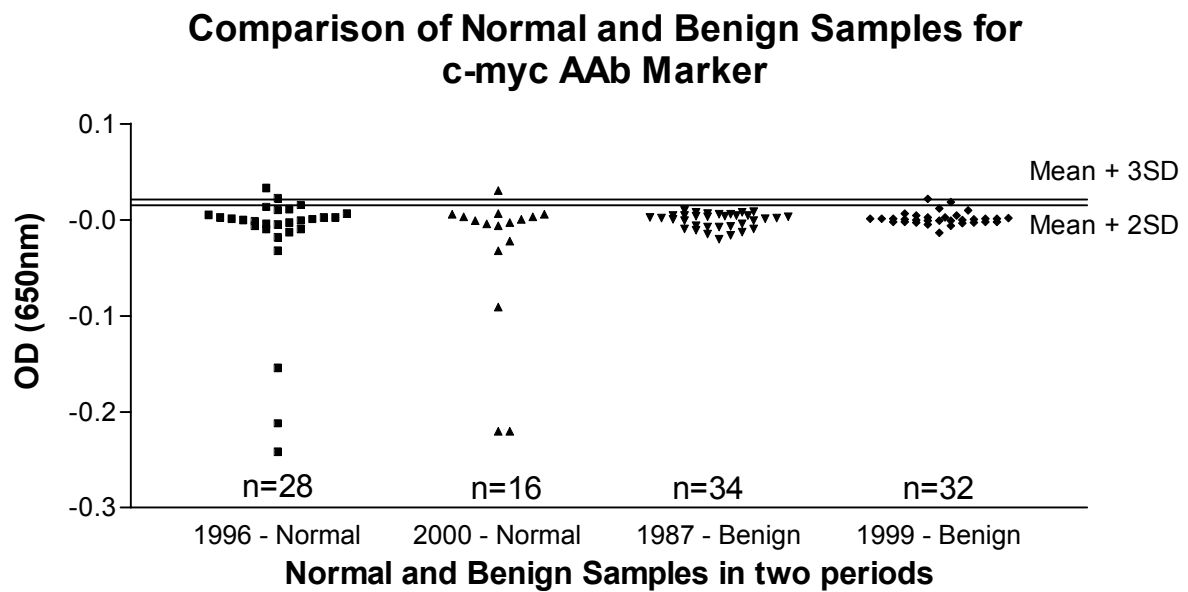
Median OD (650nm) 1987 - -0.0008333 (Range -0.01633- 0.0200) N=34

Median OD (650nm) 1999 - -0.001833 (Range -0.04833- 0.01233) N=32

P = 0.4568 (non significant)



Fig.7.6. Comparison of older and more recent Normal and Benign samples for c-myc AAb



Normal:

Median OD (650nm) 1996 - -0.0006667 (Range -0.2420 - 0.0330) N=28

Median OD (650nm) 2000 - -0.002167 (Range -0.2207- 0.0300) N=16

P = 0.6256 (non significant)

Benign:

Median OD (650nm) 1987 - 0.001667(Range -0.02033- 0.009667) N=34

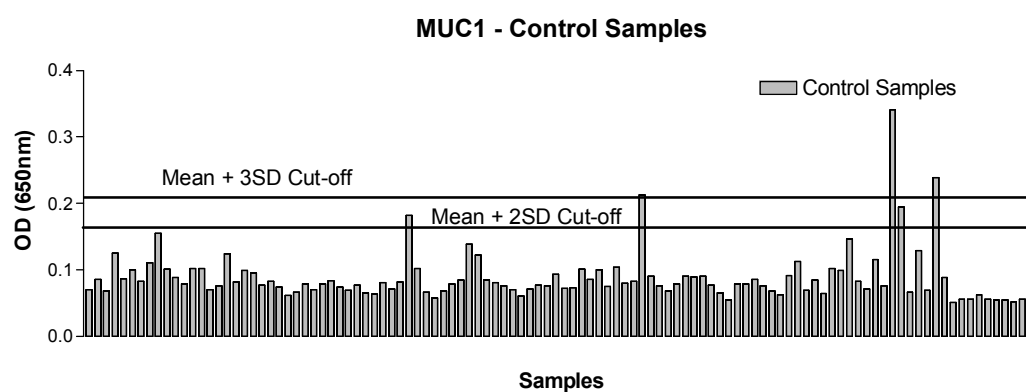
Median OD (650nm) 1999 - 0.0001667 (Range -0.0140- 0.02167) N=32

P = 0.9488 (non significant)

## 7.4. CUT-OFF VALUES

### 7.4.1. MUC1 AUTOANTIBODY CUT-OFF

Fig.7.7. Values of individual cases for all Control samples for MUC1 AAb



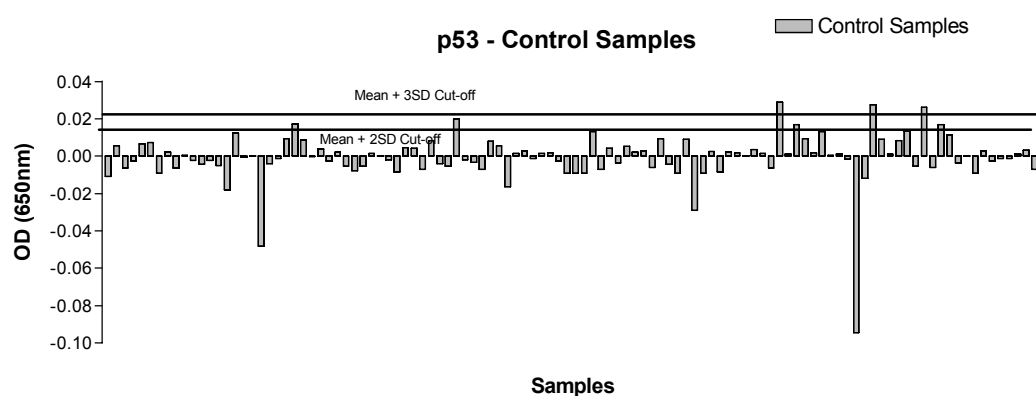
The cut-off positive value for MUC1 AAb was taken as the value greater than Mean plus 2 SD. A second positive cut-off was also calculated at the Mean plus 3SD

Table 7.7. Both Mean plus 2SD and Mean plus 3SD cut-off values of MUC1 AAb

Marker	Mean+ 2SD Mean OD (650nm)	Mean + 3SD Mean OD (650nm)
MUC1 AAb	0.167	0.208

## 7.4.2. P53 AUTOANTIBODY CUT-OFF

Fig.7.8. Values of all the individual cases of Controls for p53 AAb



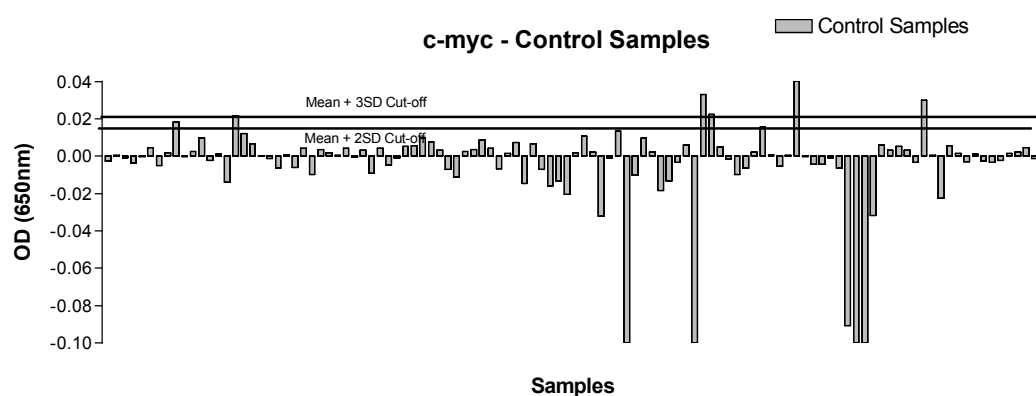
In calculating the mean values for p53 AAb, all negative differences between the antigen and VOL were taken as 0. This was since negative values denoted higher signal for the VOL control compared to the antigen and therefore implied no signal from the antigen itself.

Table 7.8. Both Mean plus 2SD and Mean plus 3SD cut-off values for p53 AAb

Marker	Mean + 2SD Mean OD (650nm)	Mean + 3SD Mean OD (650nm)
p53 AAb	0.016	0.022

### 7.4.3. C-MYC AUTOANTIBODY CUT-OFF

Fig.7.9. Values of all the individual cases of Controls for c-myc AAb



In calculating the mean values for c-myc AAb all negative differences between the antigen and VOL were taken as 0. This was since a negative value denoted higher signal for the VOL control compared to the antigen and therefore implied no signal from the antigen itself.

Table 7.9. Both Mean plus 2SD and Mean plus 3SD cut-off values for c-myc AAb

Marker	Mean + 2SD Mean OD (650nm)	Mean + 3SD Mean OD (650nm)
c-myc AAb	0.015	0.021

## 7.5. DISCUSSION

In our study most of the populations sampled were not Normally distributed as confirmed on Normality Testing (Table 7.2). Therefore for all comparison analysis medians of groups were compared by the Mann-Whitney non-parametric test for significance.

In the original protocol (Cheung 2001), both benign and normal samples were added together as the control population. This was feasible as published literature had shown minimal AAbs to MUC1, p53 and c-myc in either population (Bjerner et al, 2002; Keohavong et al, 2004; Balogh et al, 2006; O'Connell et al, 1998). Addition of both populations enabled a larger control group and therefore more accurate measurement of cut-off values.

For our thesis we adhered to the original study by incorporating both Normal and Benign groups as our control population. The two populations had similar age and sex distribution and neither was at-risk nor did they have any evidence of a cancer. Although their sample age differed substantially, it did not prove to be statistically relevant as the median values for the 3 AAbs did not differ between the older sample and the more recent ones (Figures 7.4, 7.5 and 7.6). The effect of sample age is also illustrated in our data with PBC samples (Figures 8.1, 8.2 and 8.3). For both MUC1 and c-myc AAbs there is no difference between the older and newer samples, but there was a significant difference for the p53 AAb, with more recent samples eliciting a higher signal. There were more than 100 samples in each group, which were separated by 10 years from collection. These results may be spurious or it may indicate that p53 AAb signals decrease with time. However there is really no reason to think that MUC1 and c-myc AAbs are more stable than the AAbs to p53. In more recent study from the unit (Chapman et al, 2007), only samples designated Normal were analysed to establish the

cut-off level. In this latter study, only the lower cut-off value of Mean + 2SD was used to establish positivity in PBC cases.

In establishing the cut-off margin for positive results we attempted to highlight two possible values (Tables 7.7, 7.8 and 7.9). Setting the positive cut-off value to greater than Mean + 3SD of our Control samples decreases the sensitivity of the technique as it may underestimate the true numbers of positive cases. It however increases the specificity of the assay for individual markers and also for the panel (Tables 9.13 and 9.14).

In screening techniques reducing false positives i.e. achieving a high specificity for the screening tool is an important aspect of the detection technique. This is because when screening whole populations even a slightly high number of false positive cases can overburden the system as large numbers of screened cases will require further investigation. Furthermore poor specificity resulting in false positive cases greatly distresses patients who are subjected to further tests.

The lower Mean + 2SD cut-off level was also used in recognition that the apparently Normal population may harbour occult cancer (Chapman et al, 2007) which may inadvertently raise the marker in this population. Although this lower level increases the sensitivity of the assay the specificity however decreases (Effendy 2005) (Table 9.13 and 9.14).

During the progress of our study we had noticed excessively high false positive values in the at-risk population (Table 9.1) using the lower cut-off value. Therefore in the later phase of our study when comparing sensitivities in PBC groups only the higher cut-off value was used.

The control population with only 110 samples was smaller than what we had originally designed although we did not do any formal power calculation to assess the exact numbers required. The low numbers in the Control group may result in error (type II) in the measurement of the true mean and hence cut-off values. We were unfortunately unable to collect further Benign or Normal samples during the study period. More recent data from the unit had successfully accumulated more Normal samples and therefore did not require combining two separate groups (Chapman et al, 2007).

Little can be deduced from the pathologies noted from those benign cases as there were only 3 in total that were positive for any one of the markers. Smoking is associated with the detection of mutated plasma p53 DNA in patients without cancer (Hagiwara 2006) and may explain one of the elevated AAb markers in the benign group. A second patient presented with nipple discharge and had a benign papilloma on histology.

In contrast to previous work (Cheung 2001), we had noted minimal AAbs at both cut-off levels for benign cases (Table 7.5). Cheung had demonstrated a panel sensitivity of 29%, with p53 AAb alone being 23% at the lower (Mean + 2SD) cut-off level. This marked difference for the same samples measured at two different periods by two different ELISA assay may highlight the improved assay as used by the current author. As detailed in chapter 6, our method aimed to reduce the background signal and hence any false positive results.

Less than 5% of our benign cases had a single marker rise with a panel showing 7.5% positivity (Table 7.5). However immunostaining of benign breast tissue by Sirotkovic-Skerlev detected mutant p53 oncoprotein in about 20% of cases (Sirotkovic-Skerlev et al, 2005). The presence of these oncoproteins can confer a two-fold increase in the likelihood of

malignant transformation. (Rohan et al, 2006). These results were however contradicted by Balogh et al (Balogh et al, 2006) who using ELISA to detect AAb to p53 in the sera of four women with benign breast disease, failed to detect any. Their results were similar to ours. Low prevalence of p53 AAb may in part be explained by the location of p53 oncoproteins, which are essentially intracellular.

Although Sirotkovic-Skerlev also noted a 100% prevalence of c-myc oncoproteins in benign tissue, using a micro array technique, Corzo et al (Corzo et al, 2006) failed to detect any c-myc oncogene in their normal and benign samples. Their findings are more consistent with ours. The probable role of c-myc oncogene amplification and overexpression in advanced breast cancer (Berns et al, 1992; Deming et al, 2000) may indicate that it is not present in benign breast disease.

MUC1 antigen expression in benign tissue is of low intensity and is restricted to apical cell surface membranes and lumen debris (Croce et al, 1997). This may have contributed to the low MUC1 AAb prevalence, which has also been confirmed by Croce (Croce et al, 1995). The heterogeneous antigenicity of the MUC1 antigen may also be a cause of under estimating the true prevalence of MUC1 serum AAbs in benign as well as malignant breast tumours when a single antigen is used in ELISA techniques as was the case with our study. Future development of the technique may be better using pooled MUC1 antigens from different patients rather than from a single patient. This may increase the yield of different antigens and epitopes and hence increase sensitivity of the assay to MUC1 AAbs.



## Chapter 8

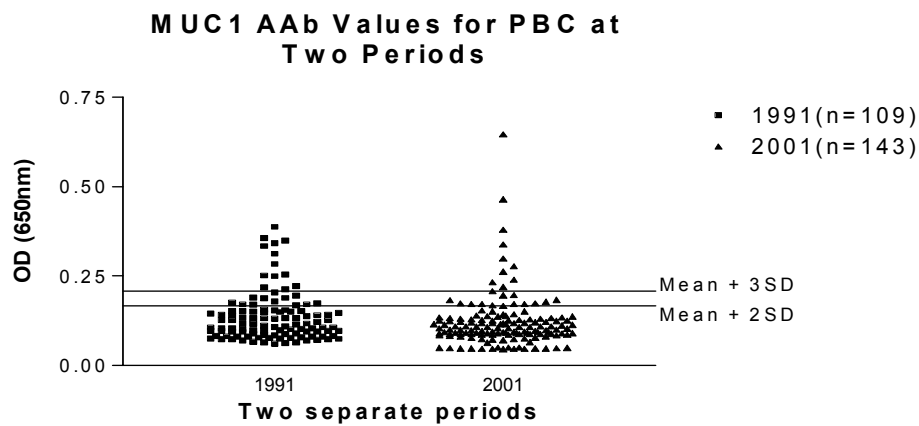
### Validation of Assay

## 8.1. ASSESSING IMMUNE RESPONSE WITH TIME

For the results of the assay to be valid we had to demonstrate that sample age did not interfere with final results as AAb signal may fade over time. Our data on Normal and Benign samples already indicated that sample age did not invalidate results as the signals attained for older samples did not significantly differ from more recent ones (Figures 7.4, 7.5 and 7.6). However to further elucidate sample age on signal strength we compared the mean OD values of larger number of samples separated by a decade.

More than one-hundred PBC samples collected in two separate periods with a 10-year gap were analysed. The PBC samples were a mixture of DCIS, screen detected and symptomatic in each period. PBC samples were used because large numbers of samples that were stored for considerable length of time (3 to 144 months) could be analysed unlike fhx samples, which had been stored for only 20 – 55 months. The median signal for the p53 AAb was reduced, as the sample was stored for longer duration. However no such pattern was seen for either MUC1 or c-myc AAbs. Indeed the signal for the c-myc AAb was borderline higher in the earlier group ( $p=0.0549$ ). It is therefore possible that the difference between the two groups might have been due to a slight difference in the types of breast cancer in each group, giving rise to different immune signatures as overall there did not appear to be a drop in the AAb signal over the storage time for these samples.

Fig.8.1. Column graph for PBC samples in two different periods for MUC1 AAb

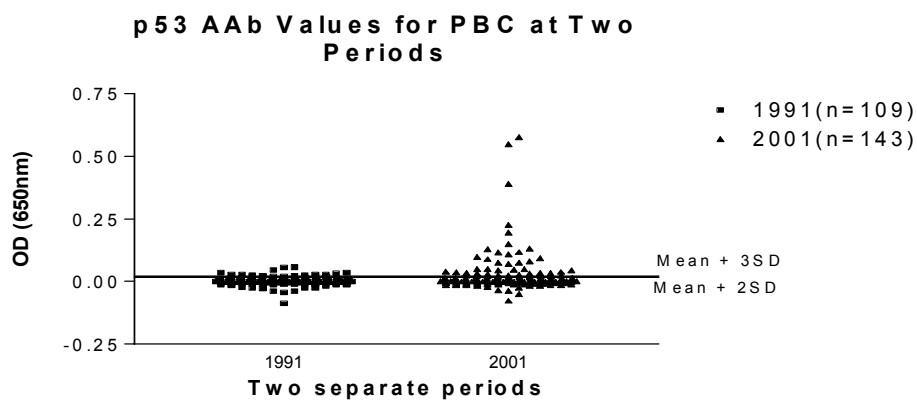


Median OD (650nm) 1991 - 0.1150 (Range 0.060 - 0.387) N=109

Median OD (650nm) 2001 - 0.1070 (Range 0.045 - 0.647) N=143

P = 0.080 (non-significant)

Fig.8.2 Column graph for PBC samples in two different periods for p53 AAb

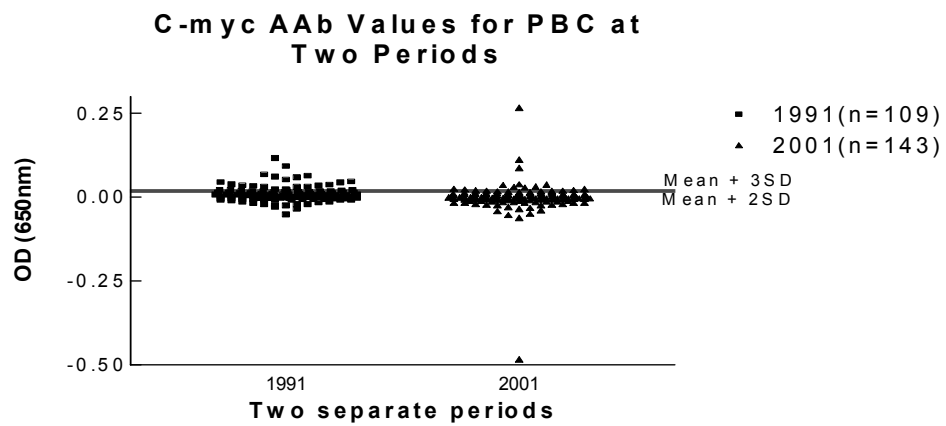


Median OD (650nm) 1991 - 0.0013 (Range -0.0873 - 0.0573) N=109

Median OD (650nm) 2001 - 0.0043 (Range -0.0763 - 0.5770) N=143

P = 0.0085 (significant)

Fig.8.3. Column graph for PBC samples in two different periods for c-myc AAb



Median OD (650nm)\_1991 - 0.0057 (Range -0.0530 - 0.1150) N=109

Median OD (650nm) 2001 - 0.0 (Range -0.4843 - 0.2667) N=143

P = 0.0549 (non-significant)

## 8.2. REPRODUCIBILITY OF ELISA ASSAY

The Bland Altman method to calculate the CR was used to assess the reproducibility of the technique.

### 8.2.1. BLAND ALTMAN COEFFICIENT OF REPRODUCIBILITY

30 random samples (10 benign, 10 fhx and 10 PBC) were analysed for the AAbs to MUC1, p53 and c-myc and then repeated once several days later. The two techniques were analysed using the Bland Altman method for the coefficient of reproducibility. The formula used was:

$$CR = 1.96 \times \sqrt{(\Sigma(d2-d1)^2 / (n-1))}$$

Fig.8.4 Bland Altman plot for the reproducibility of MUC1 AAb

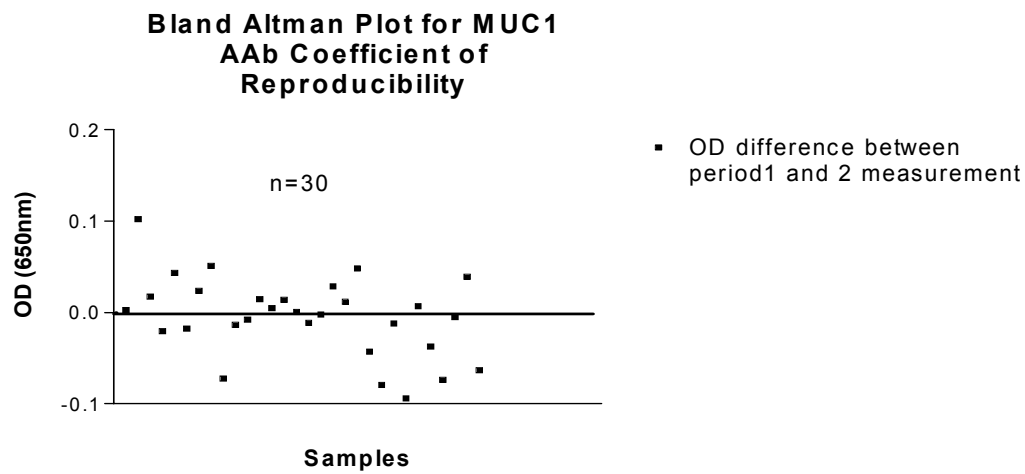


Fig.8.5 Bland Altman plot for the reproducibility of p53 AAb

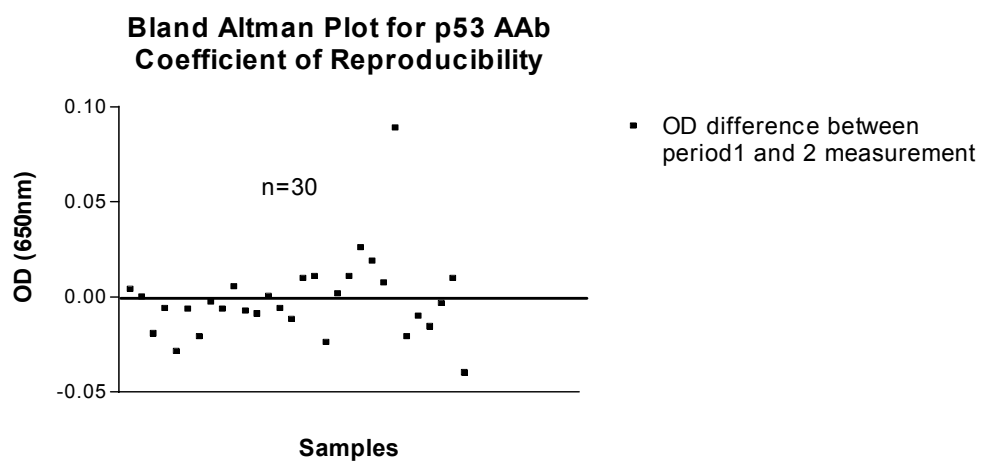


Fig.8.6 Bland Altman plot for the reproducibility of c-myc AAb

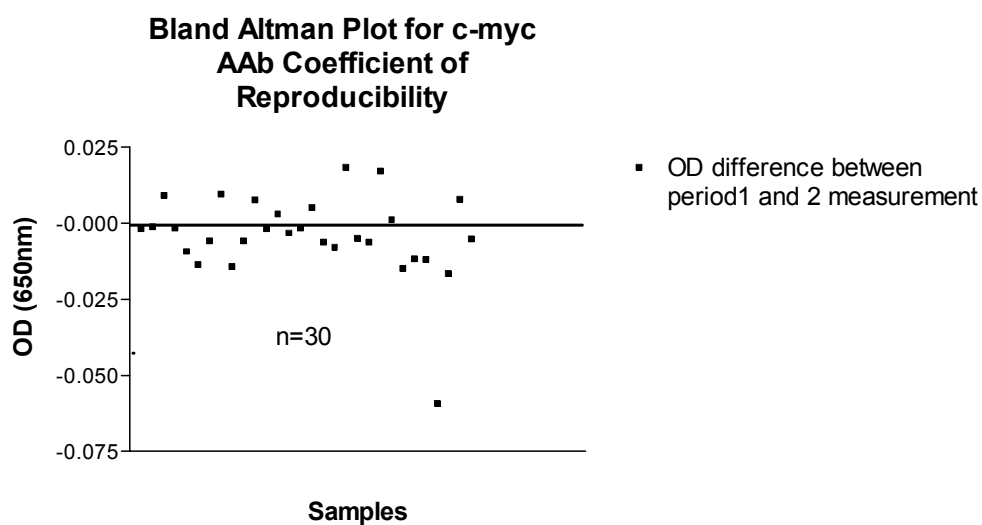


Table 8.1. Bland and Altman CR for the 3 AABs

Population	Mean OD (1)	Mean OD (2)	SD	CR
MUC1 AAb (total)	0.0987	0.0934	0.043	0.086
Benign	0.0851	0.094533	0.018	0.035
Fhx	0.1038	0.114967	0.048	0.093
PBC	0.1073	0.070767	0.043	0.084
p53 AAb (total)	-0.00032	-0.00182	0.022	0.046
Benign	0.000333	-0.00217	0.011	0.022
Fhx	0.005	-0.0031	0.011	0.022
PBC	-0.0063	-0.0002	0.035	0.069
c-myc AAb (total)	0.003856	-0.00044	0.013	0.028
Benign	0.006	0.001367	0.008	0.016
Fhx	0.002	-0.0016	0.008	0.016
PBC	0.008967	-0.0011	0.020	0.040

The CR indicates 95% of repeated values for each of the 3 markers varied by the CR value or less.

### 8.3. COMPARISON OF AUTOANTIBODY SENSITIVITY IN PBC AND CONTROL GROUPS

Four hundred and seven samples of 370 patients with known PBC were analysed for all 3 AAbs. Comparison of AAb sensitivities between the PBC group and the Control population was performed to assess the reliability of the assay.

The mean age of the PBC patients was 62.9 years (31 – 94) and mean age of the samples was 69.2 months (3 – 144 months). The mean age of the Control group was 58.8 years (34 – 90) and the mean age of the samples was 108 months (38 – 200).

Table 8.2. Comparison of AAbs in PBC and Control groups at Mean + 2SD cut-off

<b>Marker</b>	<b>+ve N (%) (PBC)</b>	<b>+ve N (%) (Control)</b>	<b>Fishers exact test</b>
MUC1 AAb	82 (20.1%)	5 (4.5%)	P < 0.0001
p53 AAb	100 (24.5%)	7 (6.4%)	P < 0.0001
c-myc AAb	65 (15.9%)	7 (6.4%)	P = 0.008
Panel of markers	199 (48.9%)	14 (12.7%)	P < 0.0001
Any 2 positive markers	44 (10.8%)	3 (2.7%)	P = 0.008
All 3 positive markers	2 (0.4%)	0 (0%)	P = 1

Table 8.3. Comparison of AAbs in PBC and Control groups at Mean + 3SD cut-off

<b>Marker</b>	<b>+ve N (%) (PBC)</b>	<b>+ve N (%) (Control)</b>	<b>Fishers exact test</b>
MUC1 AAb	42 (10.3%)	3 (2.7%)	0.012
p53 AAb	85 (20.9%)	3 (2.7%)	<0.001
c-myc AAb	40 (9.8%)	5 (4.5%)	0.088
Panel of markers	143 (35.1%)	10 (9.1%)	P < 0.0001
Any 2 positive markers	16 (3.9%)	2 (1.8%)	P = 0.387
All 3 positive markers	2 (0.4%)	0 (0%)	P = 1



Fig.8.7. Comparison of Control and PBC populations distribution for MUC1 AAb

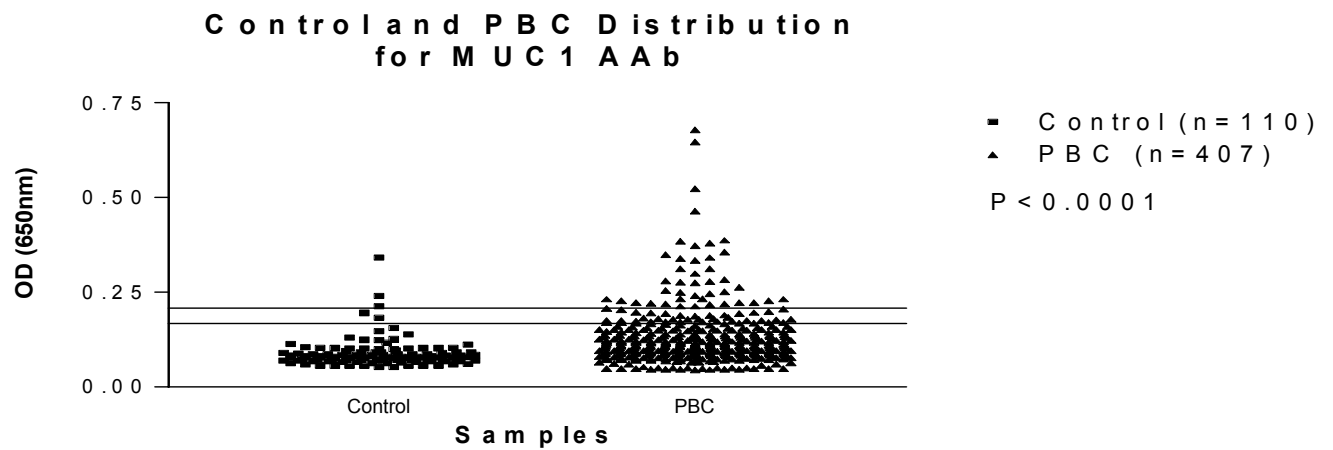


Fig.8.8. Comparison of Control and PBC populations distribution for p53 AAb

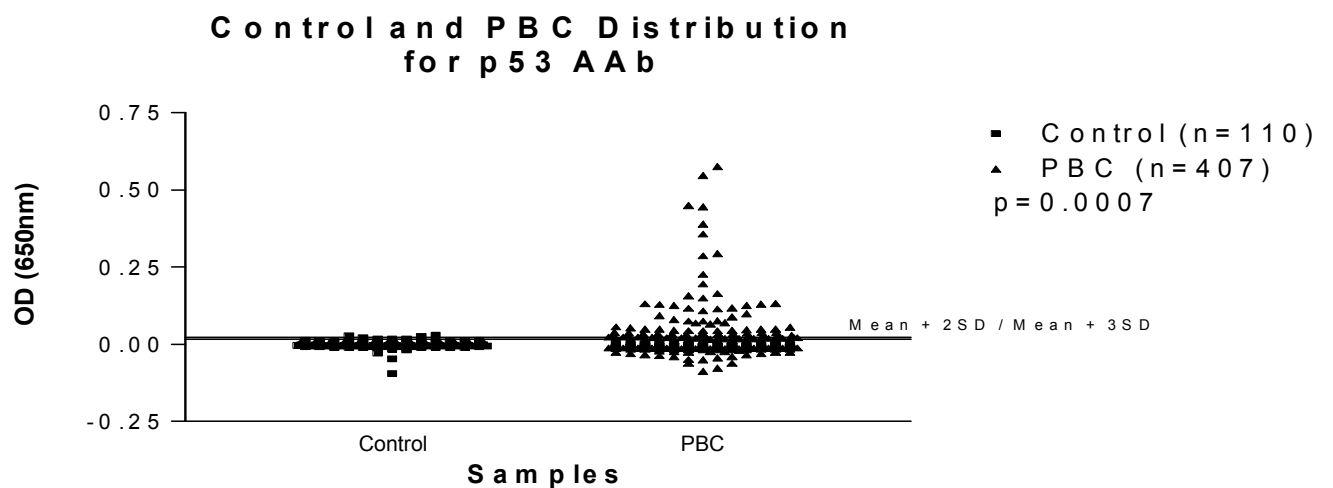
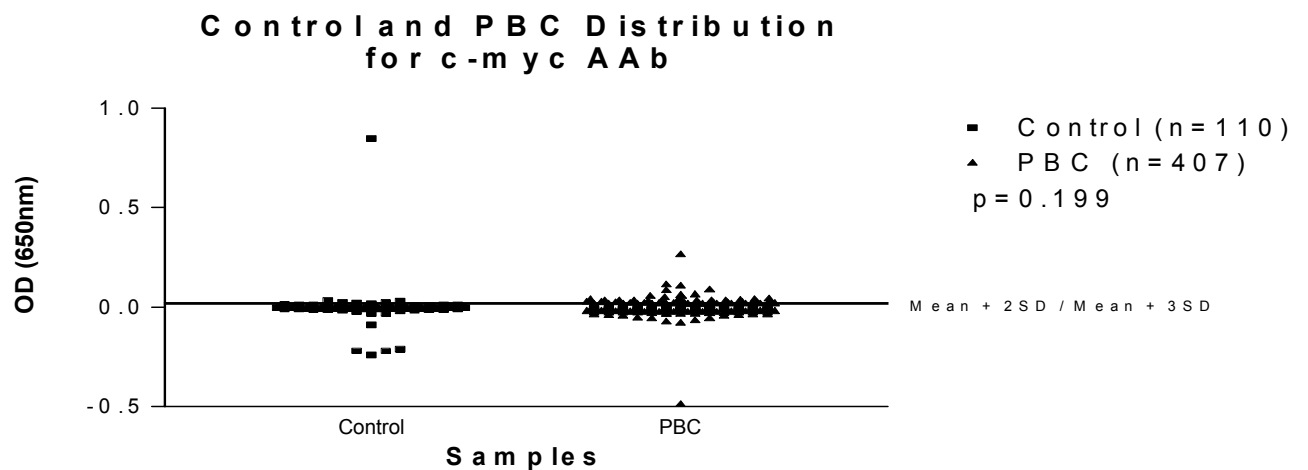


Fig.8.9. Comparison of Control and PBC populations distribution for c-myc AAb



## 8.4. DISCUSSION

Our data has demonstrated that for the AAbs to both MUC1 and c-myc, sample age did not significantly alter the signals attained. Therefore comparing data between samples of varying age was justified. However for the p53 AAb the data showed significantly lower signal for the older sample batch. It may be a spurious result in view of only 100 samples in each group. Although no power calculation was done to establish the correct numbers required for accurate assessment the current number appear to be too low. This is intuitive as there is no reason why only p53 signal is reduced i.e. p53 AAb is less stable compared to MUC1 and c-myc AAbs.

We assessed reproducibility of the assay by calculating the CR of each of the 3 AAbs using the Bland Altman method. Our results showed for the MUC1 AAb an overall CR of 0.0855, for p53 AAb CR = 0.0457 and for c-myc AAb = 0.0282 i.e. 95% of repeated values for each of the 3 markers varied by the CR value or less (Table 8.1). Interestingly within the subgroups of samples assayed, the Benign samples were consistently most reproducible for each of the 3 AAbs (Table 8.1). The reason for benign samples to be consistently reproducible is not well understood. Long duration of storage may cause a dampening of signaling, although this was not our observation when comparing the means of samples donated in 1987 and those taken in 1996. Both sets of samples gave similar signals for all three markers.

Variations in reproducibility as noted by the CR values being greater than the Mean + 2SD cut-off for p53 and c-myc AAbs indicate non-specific binding of antibodies to the VOL in the wells. Hence CR for p53 and c-myc AAbs are higher than that of MUC1 AAb, an assay that

did not use the VOL. Ten percent of human serums contain natural AAbs to biotinylated proteins (Dale et al, 1994), therefore some of the excess signal from the negative control may also be due to specific binding of antibodies to the biotin tag on VOL. Neutravidin also contributes to non-specific binding with human serum and therefore causes increased background signal in the assay.

Further causes for high background signal and therefore poor reproducibility may be that some of the samples were themselves inherently unstable due to repeated thawing and freezing. Any true signal from MUC1, p53 and c-myc AAbs is much lower in comparison to the VOL; therefore even minimal variation of reproducibility may obscure these true positive or negative results.

Although it is difficult to make final conclusions with regards to the reproducibility of our assay from these results we may infer that the ELISA developed for this study is fairly reproducible as a research tool, but there is some variability in reproducibility for differing populations and tumour markers analysed. Its use within a clinical context is however limited by this variation in reproducibility.

Further validation of the assay was assessed by comparing prevalence of markers in Control and PBC groups. At the lower cut-off all 3 markers were higher in PBC compared to Control (Table 8.2). At the higher cut-off only c-myc failed to reach significant levels (Table 8.3). However the trend appeared to support the hypothesis that tumour markers were present in sufficient quantity in those with PBC compared to Control population. This is further illustrated by figures 8.7, 8.8 and 8.8. The results therefore support our conclusion that the newly developed assay did measure tumour markers as AAbs to MUC1, p53 and c-myc.

## Chapter 9

### At-Risk Cases

## 9.1. AT-RISK

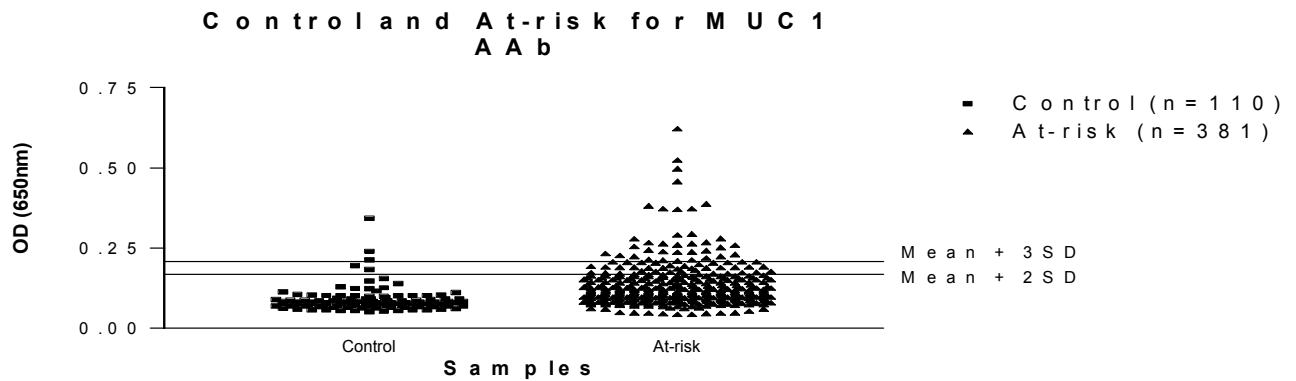
The At-risk group included 381 patients, 356 were known to have a fhx of breast cancer and 25 women with histological diagnosis of ADH. The former group included 20 women who were known to have BRCA1/BRCA2 gene mutation.

The overall frequencies of AAbs in the At-risk population for both cut-off values are presented in Table 9.1. and figures 9.1, 9.2 and 9.3.

Table 9.1.

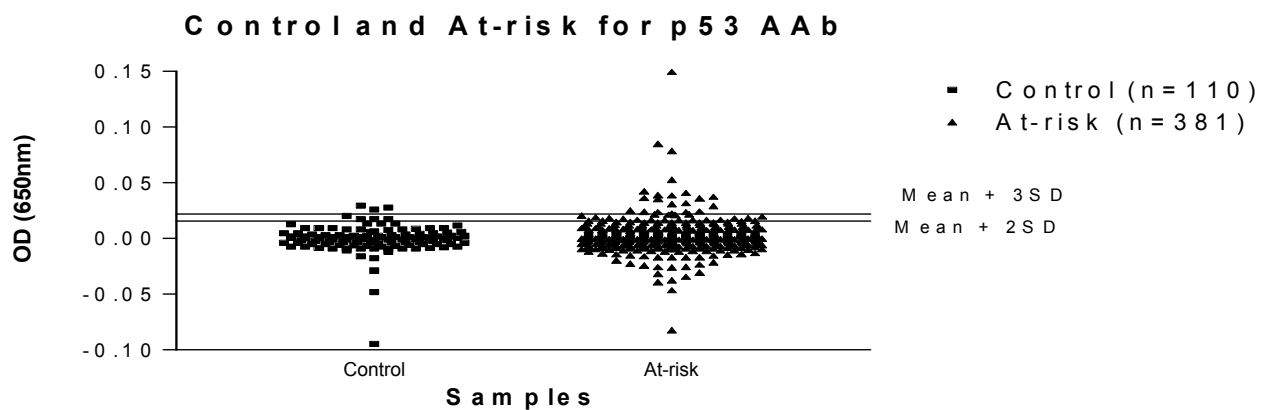
<b>Marker</b>	<b>+ve N (%)</b>	<b>+ve N (%)</b>
	<b>Mean + 2SD</b>	<b>Mean + 3SD</b>
MUC1 AAb	75 (19.6%)	36 (9.4%)
p53 AAb	42 (11%)	21 (5.5%)
c-myc AAb	33 (8.7%)	19 (4.9%)
Panel of markers	120 (31.5%)	70 (18.4%)

Fig.9.1. Comparison of at-risk and control populations for MUC1 AAb



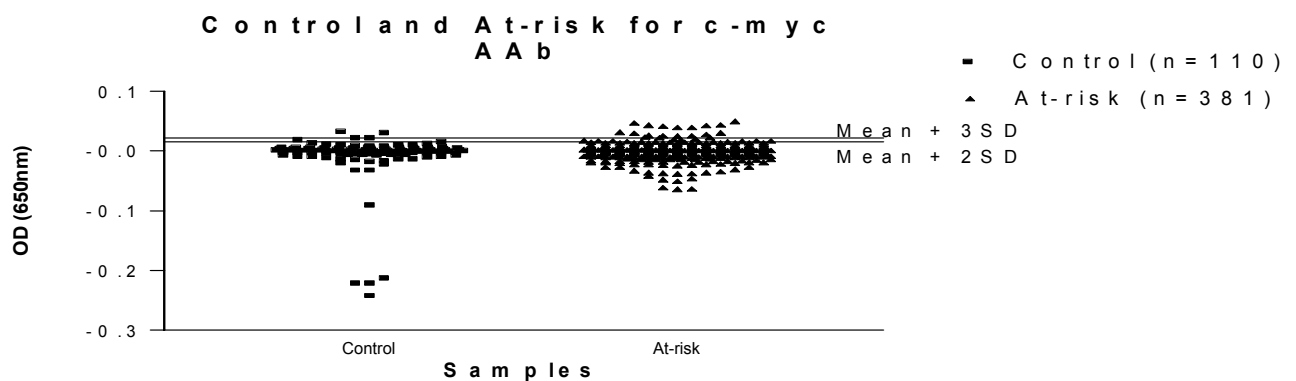
Control (N = 110) median = 0.089: At-risk (N = 381) median= 0.134  $P < 0.0001$

Fig.9.2. Comparison of at-risk and control populations for p53 AAb



Control (N = 110) median = -0.00069: At-risk (N = 381) median = 0.00276  $P = 0.0394$

Fig.9.3. Comparison of at-risk and control populations for c-myc AAb



Control (N = 110) median = -0.00129: At-risk (N = 381) median = 0.00127  $P = 0.998$

### 9.1.1. COMPARISON WITH PREVIOUS WORK

Data from previous work at the Unit (Cheung, 2001) had noted higher p53 AAb sensitivities at both cut-off levels (25.8% and 15.1% for Mean + 2SD and Mean + 3SD levels respectively). The sensitivities for the MUC1 AAb were higher in our study but for the c-myc AAb and the panel, the results were similar between the two studies.

### 9.2. SUB-GROUP ANALYSIS OF AT-RISK POPULATION

In this subgroup of at-risk population, although data is presented for both cut-off values, the analysis of the data is done using only the higher value (Mean + 3SD). The higher cut-off value provided the best possible specificity. In screening at-risk population, detection of AAbs using cut-off values to achieve the highest specificity is important.

#### 9.2.1. FAMILY HISTORY SAMPLES

##### 9.2.1.1. RESULTS FOR ALL FAMILY HISTORY CASES

356 samples were analysed of 346 patients (10 patients had 2 samples). Median follow up was 44.9 months. 253 patients were stratified into low (4 patients), moderate (97 patients) and high risk (152 patients of whom 20 were known BRCA 1 or BRCA2 gene carriers). Risk stratification was based on the Units protocol (Section 5.3.2.1). All patients at time of sampling were excluded of breast pathology by routine examination and mammogram.

Table 9.2. AAb frequencies for both cut-off values for all fhx samples

<b>Marker</b>	<b>+ve N (%)</b> <b>Mean + 2SD</b>	<b>+ve N (%)</b> <b>Mean + 3SD</b>
MUC1 AAb	66 (18.5%)	31 (8.7%)
p53 AAb	39 (10.9%)	21 (5.9%)
c-myc AAb	32 (8.9%)	19 (5.3%)
Panel of markers	117 (32.8%)	64 (18%)
Any 2 positive markers	18 (5%)	6 (1.6%)
All 3 Positive markers	1 (0.3%)	0 (0%)

#### 9.2.1.2. FAMILY HISTORY RISK CATEGORIES

Table 9.3. Frequency of markers in different fhx risk groups as either individuals or panel

(Low risk not shown as only 4 patients in the category)

<b>Marker</b>		<b>N</b>	<b>Mean + 2SD</b>	<b>Mean + 3SD</b>
MUC1 AAb	High	152	28 (18.4%)	15 (9.8%)
	Moderate	97	20 (20.6%)	9 (9.3%)
p53 AAb	High	152	12 (7.9%)	8 (5.2)
	Moderate	97	12 (12.3)	6 (6.2%)
c-myc AAb	High	152	12 (7.9%)	7 (4.6%)
	Moderate	97	10 (10.3%)	5 (5.1%)
Panel of markers	High	152	46 (30.2%)	29 (19%)
	Moderate	97	37 (38%)	18 (18.5%)



### 9.2.1.3. BRCA1/2 GENE CARRIERS

Mutated BRCA1 and BRCA2, breast cancer susceptibility genes, are proven risk factors for breast cancer (King et al, 2003; Ford et al, 1998). More than 500 mutations have been described in the BRCA1 gene (chromosome 17) and 250 have been described in the BRCA2 gene (chromosome 13) (Coughlin et al, 1999. Review). The mutations occurring at either end of the BRCA1 gene are associated with more aggressive tumours; those occurring at the 5' extremity are associated with breast and ovarian cancers, while those closer to the 3' end are associated with only breast cancer. The prevalence of BRCA1 in the general population is 0.1%. The gene is encountered in 3% of the breast cancer population and in 70% of women with inherited early-onset breast cancer. Up to 50-87% of women carrying a mutated BRCA1 gene develop breast cancer (Easton et al, 1995). Risks for ovarian (Easton et al, 1995) and prostate cancers (Douglas et al, 2007) are also increased in carriers of this mutation.

BRCA2 mutations result in breast cancer in 45% of the carriers and only 11% of ovarian cancers in this group of carriers (Antoniou et al, 2003). BRCA2 is also a risk factor for male breast cancer; carriers have a lifetime risk of 6% for developing the cancer (Syrjakoski et al, 2004). BRCA2 mutations are associated with other types of cancers, such as prostate (Tryggvadottir et al, 2007), pancreatic (Couch et al, 2007), fallopian tube (Finch et al, 2006) and peritoneum (Finch et al, 2006).

### 9.2.1.3.1. RESULTS FOR BRCA1/2

20 patients were known to carry either the BRCA1 or BRCA2 gene.

Table 9.4. Frequency of the markers in this subgroup of high-risk population

Group	Marker	+ve N (%)	+ve N (%)
		Mean + 2SD	Mean + 3SD
BRCA positive (N = 20)	MUC1 AAb	4 (20%)	1 (5%)
	p53 AAb	0 (0%)	0 (0%)
	c-myc AAb	0 (0%)	0 (0%)
	Panel of marker	4 (20%)	1 (5%)

Fig.9.4. Comparison of BRCA subgroup with Control population for MUC1 AAb

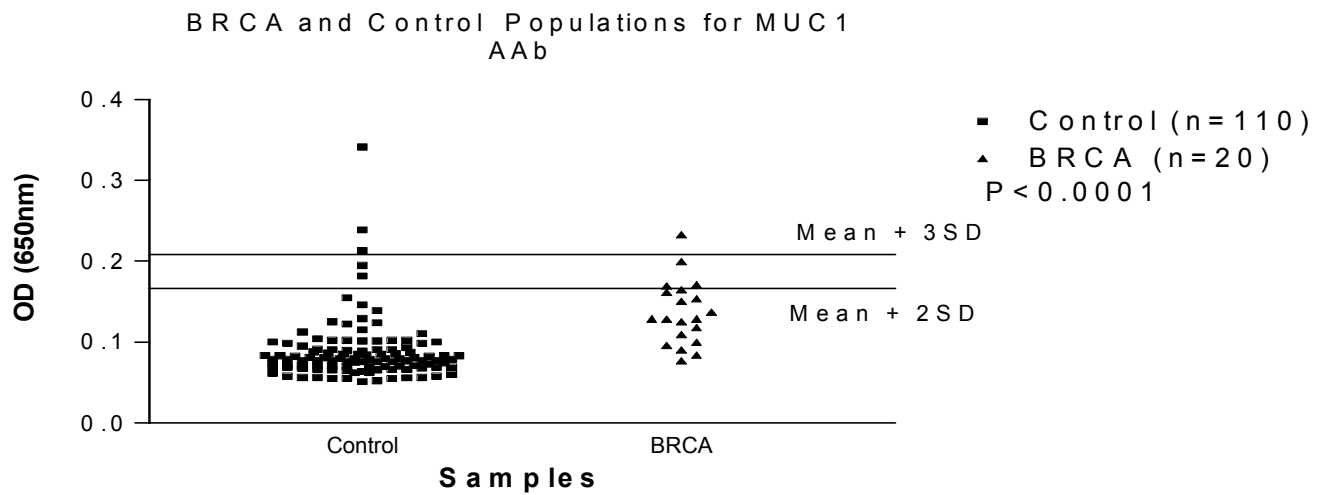


Fig.9.5. Comparison of BRCA subgroup with Control population for p53 AAb

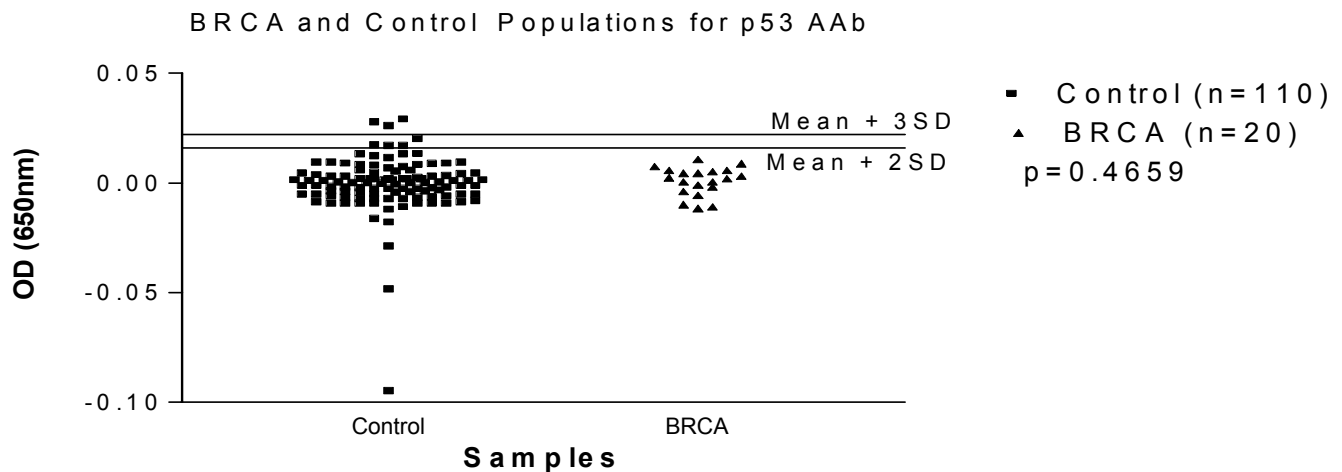
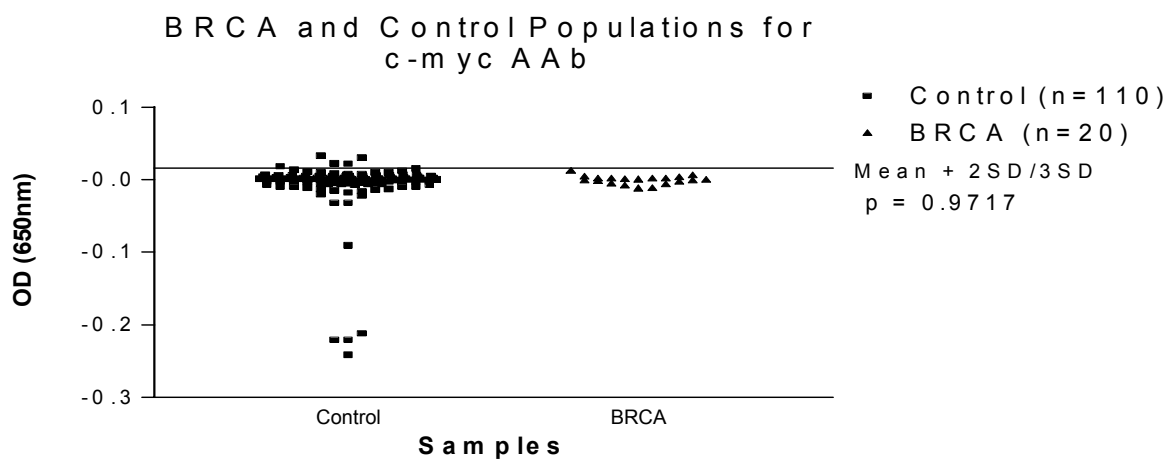


Fig.9.6. Comparison of BRCA subgroup with Control population for c-myc AAb



### 9.2.2. ATYPICAL DUCTAL HYPERPLASIA

ADH is regarded as a marker of generalized increase in breast cancer risk (Collins et al, 2007). It is associated with concomitant pre-invasive disease (DCIS) in about 10% (Renshaw et al, 2001) and is itself associated with a 3-fold increase risk in developing invasive breast cancer in the ipsilateral breast (Collins et al, 2007). It is therefore regarded as high risk. Histologically, it is a midpoint between benign proliferative breast diseases such as usual ductal hyperplasia and pre-invasive cancer i.e. DCIS. It differs from DCIS in its reduced amount of tissue involvement although the architectural and cytological features are similar to DCIS.

#### 9.2.2.1. RESULTS

Twenty-five patients whose blood samples were available for analysis were followed up due to previously diagnosed ADH.

Table 9.5. Frequency of markers in ADH subgroup

Group	Marker	+ve N (%)	+ve N (%)
		Mean + 2SD	Mean + 3SD
ADH (N = 25)	MUC1 AAb	8 (32%)	5 (20%)
	p53 AAb	0 (0%)	0 (0%)
	c-myc AAb	1 (4%)	0 (0%)
	Panel of markers	9 (36%)	5 (20%)

Fig.9.7. Comparison of ADH and Control populations for MUC1 AAb

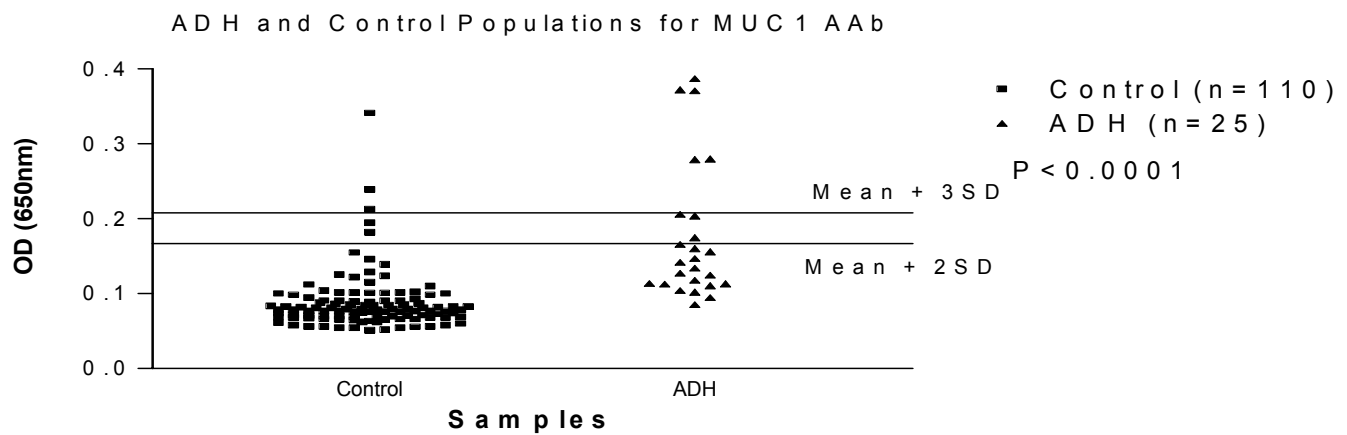


Fig.9.8. Comparison of ADH and Control population for p53 AAb

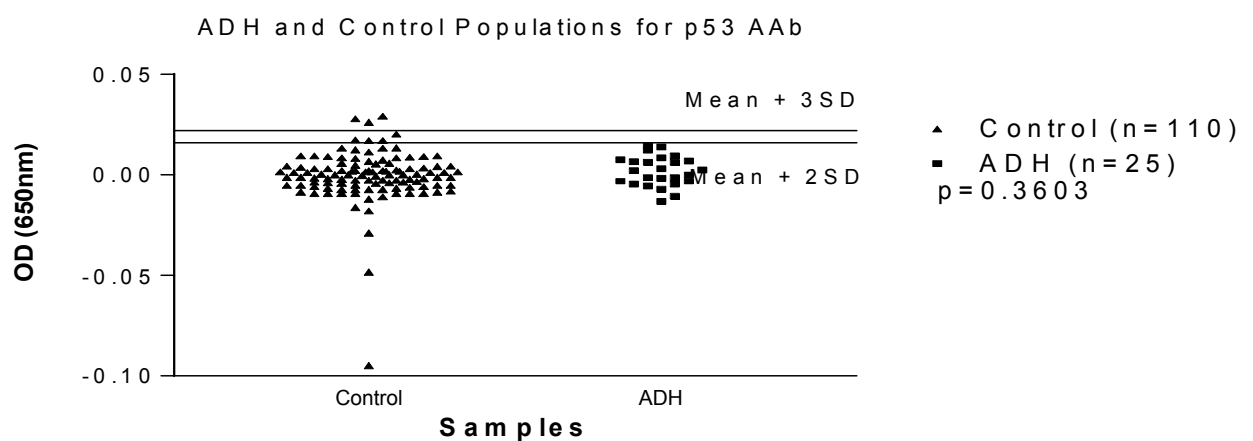
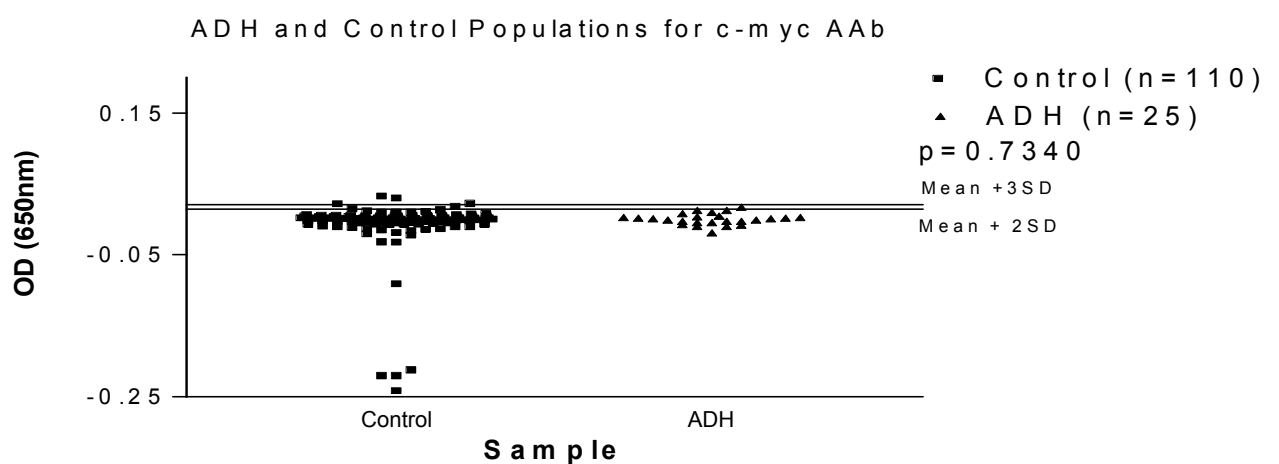


Fig.9.9. Comparison of ADH and Control population for c-myc AAb



### 9.3. AUTOANTIBODY FREQUENCY AND RISK CATEGORIES

#### 9.3.1. HIGH RISK VERSUS NON-HIGH RISK GROUPS

The at-risk group was subdivided into high risk and non-high risk for the purpose of this study. The former included all fhx cases with hospital protocol defined high risk, BRCA carriers and ADH cases. The latter group included moderate and low risk fhx cases.

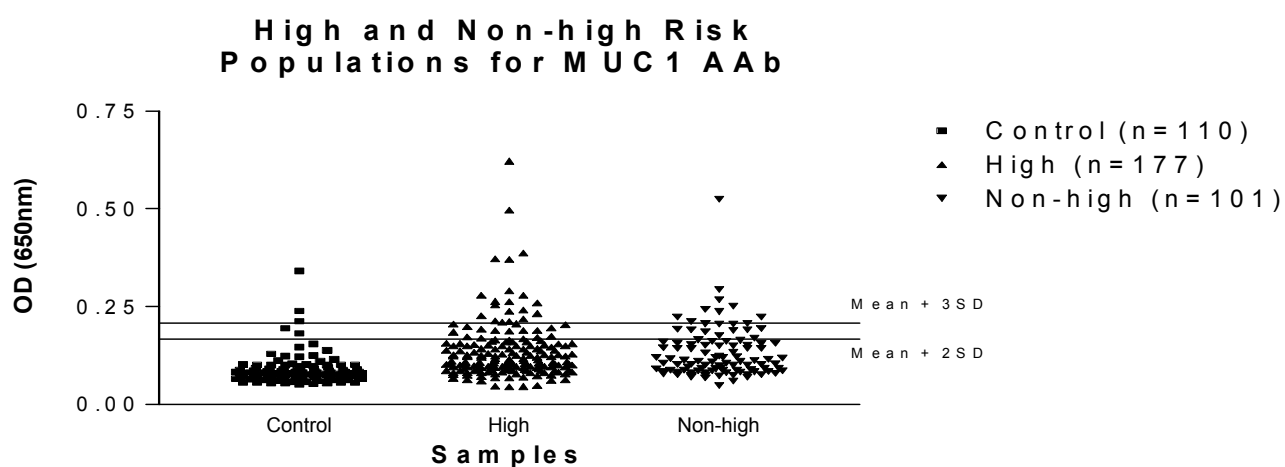
##### 9.3.1.1. MUC1 AUTOANTIBODY

Table 9.6. Correlation between MUC1 AAb and Risk Categories (Mean + 3SD Cut-off value)

MUC1 AAb	High risk (N)	Non-high risk (N)	Total (N)
Absent (N)	157	92	249
Present (N)	20	9	29
Total (N)	177	101	278

P = 0.6839 (non-significant) (Fishers exact test for significance)

Fig.9.10. Distribution of samples in each of the risk group for MUC1 AAb



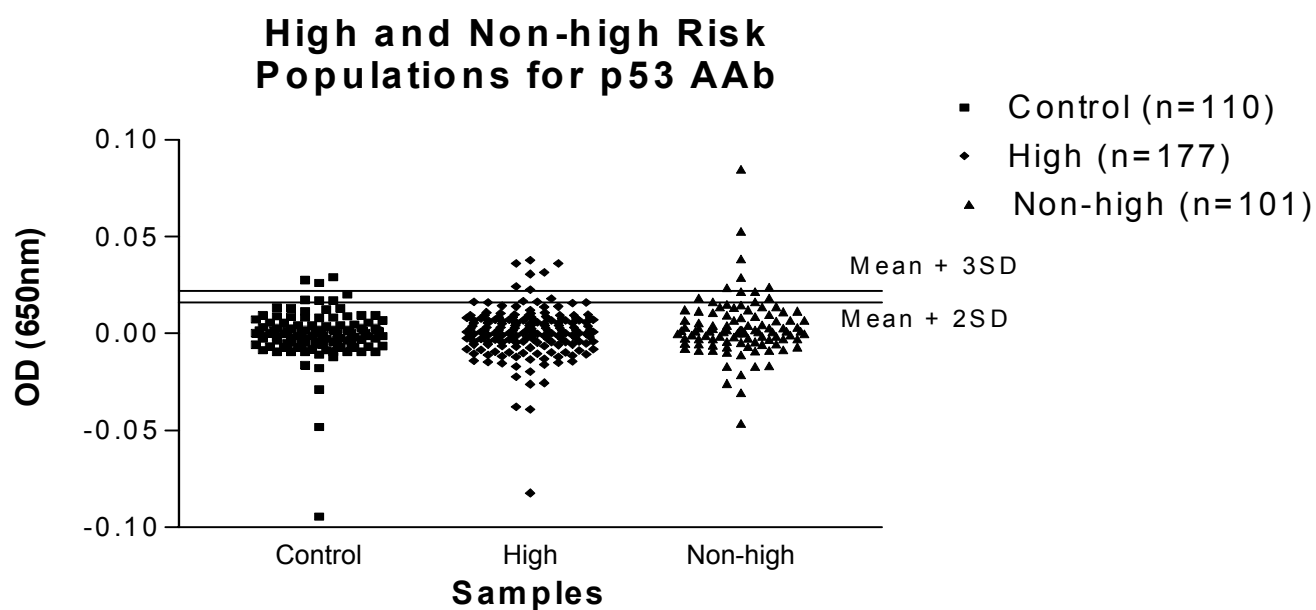
### 9.3.1.2. P53 AUTOANTIBODY

Table 9.7. Correlation between p53 AAb and Risk Categories (Mean + 3SD Cut-off value)

p53 AAb	High risk (N)	Non-high risk (N)	Total (N)
Absent (N)	169	95	263
Present (N)	8	6	14
Total (N)	177	101	278

P = 0.5829 (non-significant) (Fishers exact test for significance)

Fig. 9.11. Distribution of samples in each of the risk group for p53 AAb



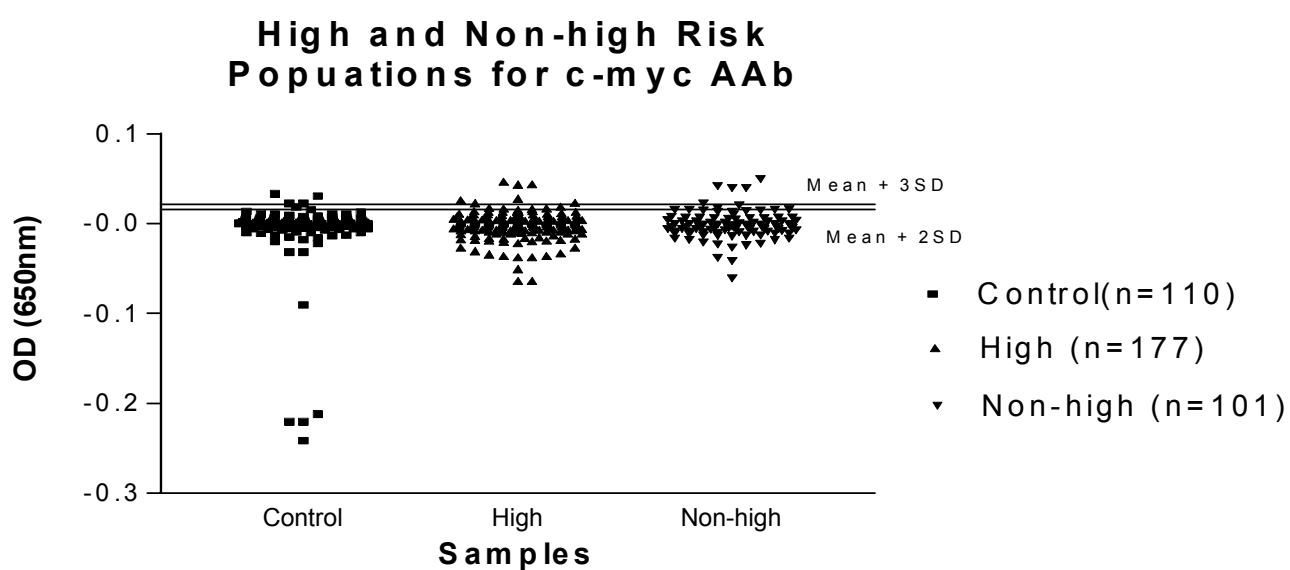
### 9.3.1.3. C-MYC AUTOANTIBODY

Table 9.8. Correlation between c-myc AAb and Risk Categories (Mean + 3SD Cut-off value)

c-myc AAb	High risk (N)	Non-high risk (N)	Total (N)
Absent (N)	170	96	266
Present (N)	7	5	12
Total (N)	177	101	278

P = 0.7624 (non-significant) (Fishers exact test for significance)

Fig.9.12. Distribution of samples in each of the risk group for c-myc AAb





### 9.3.2. COMPARISON WITH PREVIOUS WORK

There is marked difference between the current data and previous work from the Unit (Cheung, 2001). Our data failed to detect any correlation between the AAb detection and risk category in the at-risk population. Cheung had however noted a significantly higher p53 and c-myc AAbs detection in the high-risk group. MUC1 AAb detection was similar to ours. The overall panel detection in the original study was tending towards significance.

### 9.4. PROGRESSION FROM NORMAL OR FAMILY HISTORY TO PBC

13 of 381 (3.4%) at-risk patients progressed to breast cancer during the study period. Seven of the tumours were pre-invasive and 6 were invasive. One other patient progressed to develop atypical lobular hyperplasia.

All 13 patients were from the fhx subgroup. None of the patients originally sub grouped as ADH or BRCA were known to have developed breast cancer at the time of writing this thesis. Eight of the patients who developed cancer were originally categorised as high-risk and 3 as non-high. We do not have data on the remaining two cases.

Table 9.9. Exact pathology of tumour of at-risk group who progressed to PBC

Sample number	Age at sampling	Age at diagnosis	Tumour type	Grade	Stage	Size	ER	VI
107	54	55	Ductal	1	3	11mm	0	No
138	43	46	Ductal	3	2	22mm	0	No
144	51	55	DCIS	High		30mm		
176	42	46	Lobular	2	1	21mm	+	0
227	54	54	Lobular	2	1	47mm	100	No
320	51	53	Tubulo-lobular	1	1	27mm	+	Probable
425	39	41	DCIS	High	0/5	40mm		
590	67	69	NST	2	0/4	15mm	-Ve	+
605	55	56	DCIS	High	None sampled	35mm		
709	50	54	NST	2	2	9mm	200	No
711	34	41	DCIS	High		40mm		
818	47	48	DCIS					
843	41	41	DCIS					

To reduce false positive cases i.e. attain a high specificity; the higher cut-off value was used to determine whether a sample was positive.

Table 9.10. Detection of AAbs in at-risk samples that progressed to PBC (Mean + 3SD)

Sample number	MUC +ve	AAb	p53 +ve	AAb	c-myc +ve	AAb	Panel	Time to diagnosis from +ve marker. (Months)
107	Negative		Negative		Negative		Negative	N/A
138	Negative		Negative		Negative		Negative	N/A
144	Negative		Negative		Negative		Negative	N/A
176	Negative		Negative		Negative		Negative	N/A
227	Negative		Negative		Negative		Negative	N/A
320	Negative		Negative		Negative		Negative	N/A
425	Negative		Negative		Negative		Negative	N/A
590	Negative		Negative		Negative		Negative	N/A
<b>605</b>	<b>Positive</b>		<b>Negative</b>		<b>Negative</b>		<b>Positive</b>	<b>8</b>
709	Negative		Negative		Negative		Negative	N/A
<b>711</b>	<b>Negative</b>		<b>Positive</b>		<b>Negative</b>		<b>Positive</b>	<b>79</b>
818	Negative		Negative		Negative		Negative	N/A
843	Negative		Negative		Negative		Negative	N/A

For all 13 patients who progressed to PBC from the at-risk group, the mean time to diagnosis from donating their sample was 18 months. Only 2 markers (in 2 patients; MUC1 and p53 AAbs) from the above samples were positive at the Mean + 3SD cut-off level prior to the diagnosis of breast cancer.

The mean lead-time from raised marker to diagnosis of breast cancer for the positive panel cases was 43.5 months. One patient had raised MUC1 AAb and the other raised p53 AAb prior to diagnosis of breast cancer. The latter individual had a lead-time of 79 months. Both patients progressed to in-situ disease i.e. DCIS. This type of breast cancer has not infiltrated beyond the basal lamina but still elicited an immune response, which was detected in our assay. At the lower cut-off value, 4 markers in 3 patients were raised with mean lead-time of 35 months. We failed to detect any positive values for c-myc at either cut-off values.

At the cut-off level required for screening i.e. Mean + 3SD, 64 at-risk patients were positive for one marker, 6 for any two markers and none of the cases were positive for all 3 markers. Only two cases (sample numbers 605 and 711) progressed to breast cancer during the study period. The number of false positive cases in the at-risk group was therefore 68. The median follow up for this group was 51.1 months (26.7 – 145.4 months).

## 9.5. SENSITIVITY AND SPECIFICITY

Sensitivity of the assay as a screening tool to detect occult tumour is shown in Tables 9.11 and 9.12. Sensitivities of the markers in each population at the higher cut-off level is shown in Table 14.1 and further summarised in Table 14.2. Specificities of the assay as individual markers and as a panel are calculated using both Control and At-risk groups. We were able to determine the true and false positives and negatives in these two groups (Tables 9.13 and 9.14).

In screening cases true positives are those that had a raised marker prior to detection of breast cancer whilst false negatives had no raised marker before tumour was diagnosed. True negatives are cases, which were negative for the marker and did not progress to cancer. False positives are cases, which were positive for the marker but did not progress to cancer.

### 9.5.1. SENSITIVITY OF ASSAY TO DETECT OCCULT TUMOUR IN SCREENING

Table 9.11. Sensitivity of assay in screening using the Mean + 2SD Cut-off Value

Marker	True Positive	False Negative	Sensitivity
MUC1 AAb	3	10	23.1%
p53 AAb	1	12	7.7%
c-myc AAb	0	13	0%
Panel of markers	3	10	23.1%
Any 2 AAbs	1	12	7.7%
All 3 markers	0	13	0%

Table 9.12. Sensitivity of assay in screening using the Mean + 3SD Cut-off Value

Marker	True Positive	False Negative	Sensitivity
MUC1 AAb	1	12	7.7%
p53 AAb	1	12	7.7%
c-myc AAb	0	13	0%
Panel of markers	2	11	15.4%
Any 2 AAbs	0	10	0%
All 3 markers	0	13	0%

### 9.5.2. SPECIFICITIES OF THE ASSAY FOR EACH MARKER AND PANEL

Using Control and At-risk samples to calculate the specificity of the markers

Table 9.13. Specificities of assay at Mean + 2SD Cut-off

Marker	True Negative	False Positive	Specificity
MUC1 AAb	402	76	84%
p53 AAb	433	45	90.6%
c-myc AAb	438	40	91.6%
Panel of markers	339	139	70.9%
Any 2 AAbs	460	18	96.2%
All 3 markers	478	0	100%

Table 9.14. Specificities of assay at Mean + 3SD Cut-off

Marker	True Negative	False Positive	Specificity
MUC1 AAb	442	36	92.4%
p53 AAb	455	23	95.2%
c-myc AAb	454	24	95%
Panel of markers	400	78	83.6%
Any 2 AAbs	471	7	98.5%
All 3 markers	478	0	100%

### 9.5.3. COMPARISON WITH PREVIOUS WORK

Previous work only noted 2 cases of at-risk that developed breast cancer during the study period. Only one case had a raised marker in the pre-diagnosed sample. This patient elicited a rise in p53 AAb 6 months prior to the diagnosis of her breast cancer. This mimics our data with regards to p53 AAb detection prior to diagnosis. There were no sensitivity and specificity calculations in previous work from Cheung (Cheung, 2001).



## 9.6. DISCUSSION

Current mammographic screening for breast cancer, although successful, has limitations that reduce its usefulness. Hence there is a need for alternative methods. Measuring autoantibodies to tumour-associated antigens as tumour markers bypasses some of these limitations such as age dependency, user dependency and potential for developing radiation related breast pathology. Furthermore serum markers need not be masked in patients who are currently on HRT.

In establishing the potential for detecting occult breast tumours using our technique, we were able to study our assays in at-risk patients. We noted significant increase in signal for both MUC1 and p53 AAbs in the at-risk compared to the Control group (Figures 9.1 and 9.2). This further validates the assay as it is able to detect early changes in the at risk group.

The c-myc AAb signal was not different in the two groups. This may be explained by MUC1 and p53 alterations noted in the early phase of cancer evolution whereas c-myc is preferentially noted in the latter phase of established carcinoma (Deming et al, 2000), hence lack of c-myc AAb signal noted in the at-risk group.

Formal risk stratification of fhx cases was only available in 71% (253/356) of our cases. Data to establish risk category for remaining 103 patients was not available to the author. Majority of those stratified were either moderate or high risk. Only a small number of patients were classified as low risk. This is presumably because those that were defined in this category were adequately managed by most GPs and therefore not routinely referred to tertiary centers

such as Nottingham City Hospital. Furthermore low risk patients were not followed up in the routine fhx clinic.

Low risk samples were not included in the control group because there was still a small but definite increased risk compared with women who do not have any fhx (Collaborative Group on Hormonal Factors in Breast Cancer, 2001).

In all fhx samples, stratified and non-stratified, a single tumour marker was detected in 18% cases at the Mean + 3SD cut-off mark but excessively high of 32.8% when using the lower cut-off (Table 9.2). Interestingly frequency of cases with 2 positive markers was only 1.6% for Mean + 3SD cut-off. The specificity at this cut-off for 2 positive cases was 98.5%. This is clinically useful but it has virtually no use in detecting occult tumour as the sensitivity is too low (Table 9.12).

In the fhx group MUC1 AAb prevalence was higher than p53 or c-myc AAbs (Mean + 2SD level). This may not be unexpected as the MUC1 antigen is secreted in low quantity by normal healthy individuals which may result in an immune response eliciting its AAb (Bjerner et al, 2002).

The array of differing MUC1 antigens that can be shed from tumours of different types may elicit various AAbs. We can therefore increase the yield of MUC1 antibody detection by using antigens extracted from more than one patient.

Patients with known BRCA1 or BRCA2 gene have an increased (80% lifetime) risk of developing breast cancer. However at the time of study, none of the 20 women with known

BRCA1 or 2 gene mutations was diagnosed with breast cancer. Only 4 BRCA cases had a positive marker at the lower cut-off and one at the higher cut-off level. The marker detected in these cases was MUC1 AAb (Table 9.4). The significance of this to detect occult tumour is unknown at this point.

Since both BRCA and p53 gene mutations are early genetic events in the development of breast cancer, p53 gene mutations are seen more commonly in BRCA1 positive cases compared to age-matched controls (Honrado et al, 2006). Lack of p53 AAb in this group in our study may therefore suggest the absence of any true occult tumours. Further follow-up in this group will confirm this hypothesis.

ADH is recognized as a marker of breast cancer. It is associated with DCIS in about 10% of cases. Our data showed that 32% of ADH cases expressed AAbs to MUC1 (Table 9.5). This correlates well with findings of 40% of ADH tissues that were immunohistologically stained to detect the MUC1 antigen in lumen of ducts (O'Connell et al, 1998). However, only a single rise in c-myc AAbs and no p53 AAbs were detected in any of the ADH serum samples.

p53 gene mutation is an early genetic event in breast cancer formation and therefore a significant detection in preneoplastic tissue is expected. Keohavong in a small study managed to detect p53 oncoproteins in 5 out of 6 ADH tissue samples (Keohavong et al, 2004). However no data is presently available on the presence of c-myc oncoprotein in ADH tissue samples. This discrepancy in p53 staining in ADH and lack of AAb detection in our study may be accounted for by the p53 oncoprotein being normally a nuclear protein and on occasions found within other cellular organelles. Such locations may inhibit an immune

response. The extra cellular MUC1 antigens may theoretically facilitate easier AAb formation.

When analyzing AAb detection and risk category, there appears to be no discernible difference between the high and non-high risk groups (Tables 9.6, 9.7 and 9.8). Lack of correlation between the risk categories and AAb marker detection differs distinctly from previous research in our unit. Cheung (Cheung, 2001) had noted higher sensitivity of p53 and c-myc AAbs in the high-risk group compared to the non-high risk group. This difference may be explained by difference in ELISA techniques used as explained in earlier section. Reducing the background signal may have accounted for the lower p53 and c-myc AAb sensitivity in our study. Results from Cheung may also have arisen by chance due to multiple testing in his study (type I error).

Table 9.9 demonstrates the cancer types of all 13 patients that progressed to breast cancer from the flx group. The spread of cancers do not appear to be typical of familial breast cancers as few were grade 3 and stage 3 (Eccles et al, 2007).

Only 2 cases out of 13 women had a raised marker prior to breast cancer diagnosis (Table 9.10) at the higher cut-off level (3 cases at the lower cut-off). The assay therefore had a low sensitivity to detect occult tumours in the at-risk population either as individual markers or as a panel (Table 9.12). Even when a lower cut-off value was used the panel sensitivity was only 23.1% (Table 9.11). This was too low for any clinical purpose although maybe promising for future developments.

The specificities (false positive rate) for all the markers individually in the at-risk group were above 80% (Table 9.13). However at the higher cut-off value the specificity increased to over 90% for all markers (Table 9.14). This was further enhanced if 2 or more markers were detected. The specificity at this level may have clinical value although the reduced sensitivity contradicts any clinical usefulness in screening

The detection of MUC1 and p53 AAbs prior to the diagnosis of breast cancer was highlighted also by Cheung (Cheung 2001). In his work, the assay had detected a raised AAbs to p53 and MUC1 (the latter only detected at the lower cut-off value) in the same patient 6 months prior to diagnosed breast cancer. The detection of these two markers signify early phase of cancer formation. Its use therefore in screening may be indicated if the assays are further refined.

Both cases of at-risk patients to progress to breast cancer with a pre-diagnosed positive marker actually progressed to in situ disease and therefore had not infiltrated beyond the basal lamina. Although the tumour cells were not invasive, an immune response was elicited resulting in the formation of MUC1 and p53 AAbs as detected in our assay. Lack of sensitivity of our markers may result from the limited number of markers used in the panel (MUC1, p53 and c-myc AAbs).

The mean time to diagnosis from sample donated of 18 months may have been too long for accurate assessment although the patients who did sero-convert prior to diagnosis of breast cancer did so with a mean lead-time of 43.5 months. One of the individuals showed AAbs to p53 some 7 years prior to diagnosis. Such long lead-time indicates that either tumour can elicit an immune response at the earliest period of carcinogenesis or that the result on this individual was coincidental. In a recent publication on lung cancer another group have

reported AAbs up to 3.5 years before the diagnosis of lung cancers (Li et al, 2005). If the lead-time is truly 7 years then making a formal diagnosis and locating the pathology may be impossible as no current investigation can detect the lesion that is 7 years from clinical diagnosis although further refinements of breast MRI may make this more realistic in the near future.

Our current assays which are prototypes using only 3 AAbs despite having a fairly high specificity for individual markers using the Mean + 3SD cut-off, lacks sensitivity and therefore do not at this stage justify its use in screening for breast cancer in the healthy but at-risk group. The use of the panel using the Mean + 2SD cut-off value also cannot be justified at this stage; as the specificity of this particular assay is too low for screening, and in this circumstance did not greatly increase the sensitivity. In order to increase the sensitivity, further research is needed in ascertaining the optimum lead-time required before the breast cancer is detectable via ELISA. This may require obtaining more samples at shorter interval.

## Chapter 10

### Sequential Samples

## 10.1. SEQUENTIAL SAMPLES

Samples from several women from two different groups were analysed for sequential data. Sequential samples were analysed to assess whether there were any normal variance in AAbs with time or effect of treatment. Ten women from the family history donated samples on more than one occasion. These samples were analysed separately from those that were part of the ATAC trial as the latter group (twenty-one women) had diagnosed breast cancer for which they were undergoing endocrine intervention. In view of the blind nature of the study, the author was not able to ascertain individual patient's exact treatment although all patients were on an antioestrogen as part of trial.

Table 10.1. Fhx sequential samples

<b>N</b>	<b>Mean age</b>	<b>Mean interval between samples (months)</b>
10	43 (32 – 49)	13 (3 – 17)

Table 10.2. PBC sequential samples

<b>N</b>	<b>Mean age</b>	<b>Mean interval between samples (months)</b>	<b>Median NPI</b>
21	68 (54 – 82)	4 (2 – 9)	3.8 (2.3 – 6.4)



### 10.1.1. SEQUENTIAL FAMILY HISTORY SAMPLES

Fig.10.1. MUC1 AAb detection in sequential samples from fhx group

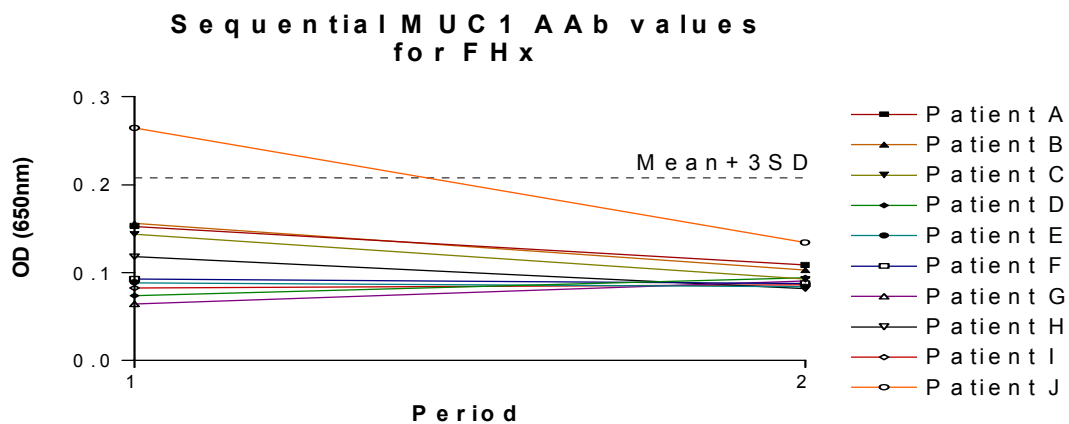


Fig.10.2. p53 AAb detection in sequential samples from fhx group

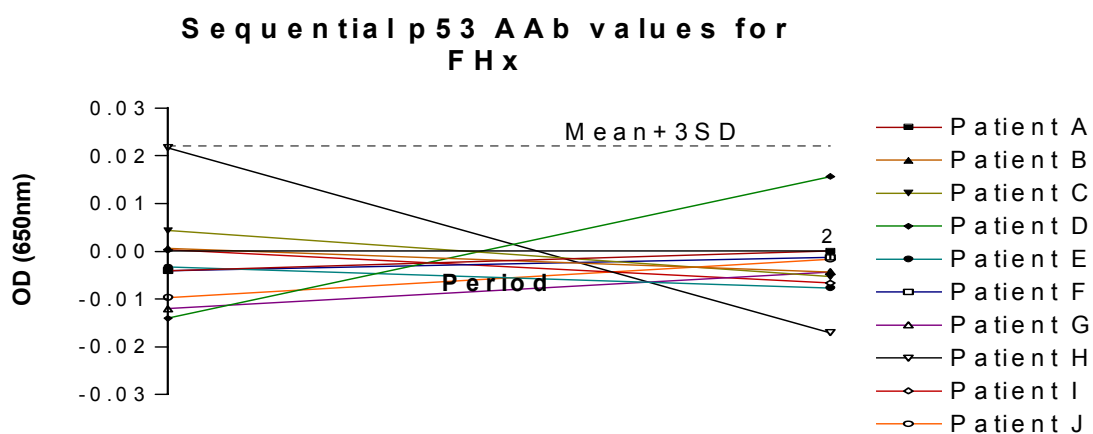
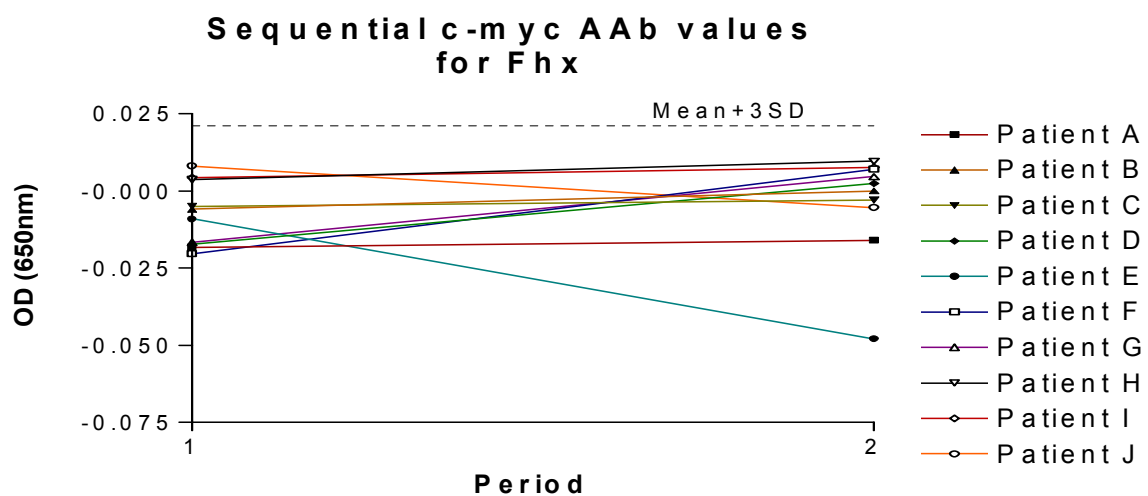


Fig.10.3. c-myc AAb detection in sequential samples from fhx group



### 10.1.2. SEQUENTIAL PBC SAMPLES

Fig.10.4. MUC1 AAb detection in sequential samples for PBC patients on ATAC trial

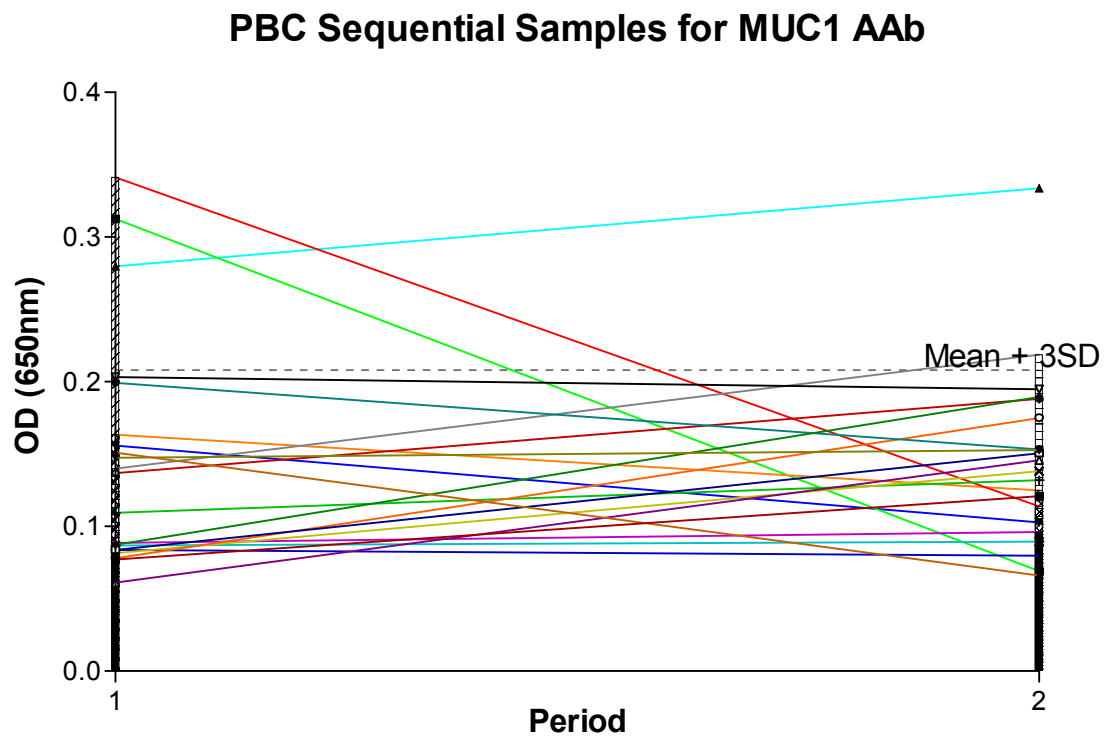


Fig.10.5. p53 AAb detection in sequential samples for PBC patients on ATAC trial

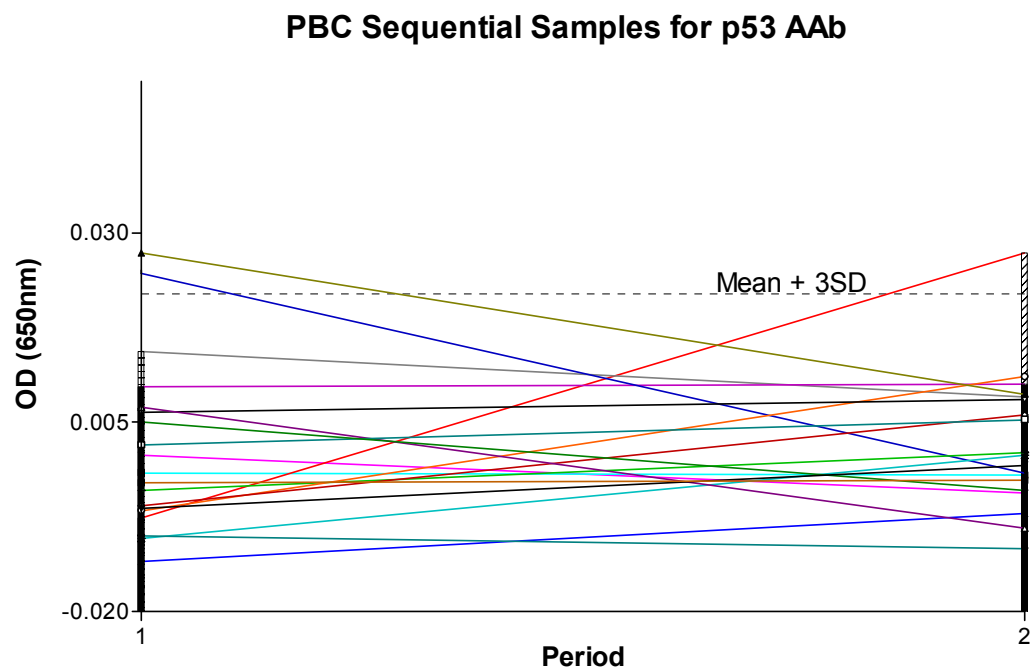
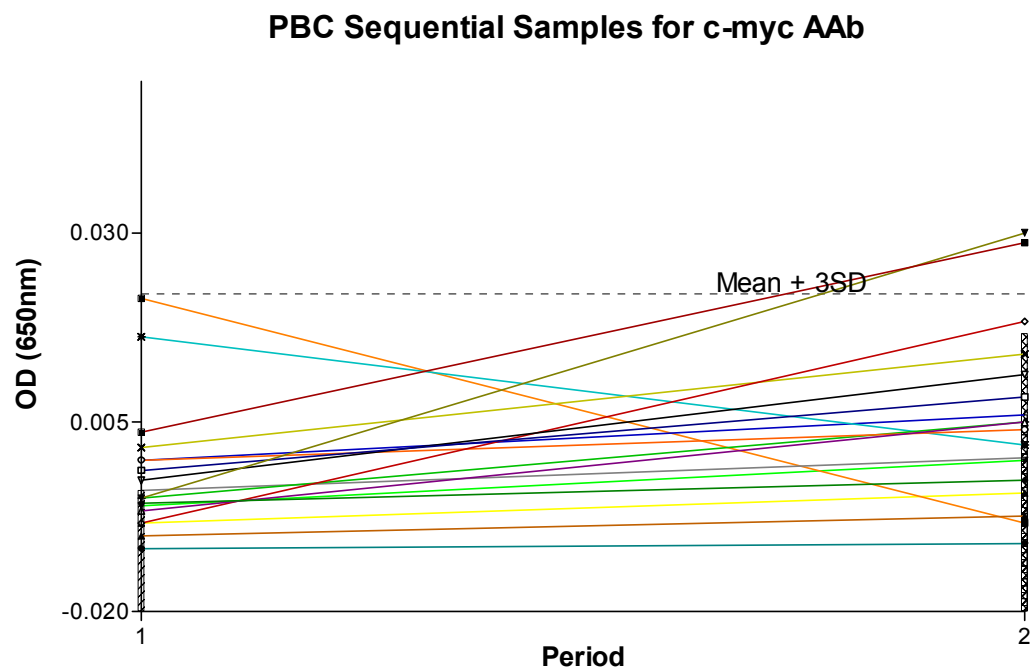


Fig.10.6. c-myc AAb detection in sequential samples for PBC patients on ATAC trial



## 10.2. DISCUSSION

10 family history at-risk women had two sequential samples donated. None of these cases had progressed to development of breast cancer at the time of writing this thesis. This is not surprising given that a maximum of 10 in every 1000 women in such an 'at-risk' group would be expected to develop breast cancer each year. In two cases a positive result was noted on initial sample but analyses of subsequent samples were deemed to be negative (Figure 10.1 and 10.2).

Most of the patients showed differences between the two sequential samples analysed. This did not reach the arbitrary cut-off for majority of the samples. These differences in sequential samples may be due to a natural variation although the low number of patients analysed make any conclusion unreliable.

The differences in signal of some of the sequential samples may also suggest that methodological problems are inherent in the assay. If so, then we are unable to overly rely on an individual finding. Group results may be more important for overall conclusion.

Since tumour marker levels in the serum may reflect overall tumour burden some markers may decrease in level in the serum after excision of the primary tumour. Sequential analysis of PBC patients may reflect this change. Our study has shown for all 3 markers, majority of patients remained negative from pre-op blood test to post op sample. For the MUC1 AAb marker, two patients became sero-negative after treatment was initiated (Figure 10.4). One patient remained positive after treatment. Even a 3-week treatment with tamoxifen can reduce MUC1 antigen levels in breast cancer tissue (Hanson et al, 2001), which may therefore

reduce the AAb levels in the serum. This may explain the two patients who became negative for the marker in their sequential sample or just indicate a natural variation of sero-conversion with no significance.

One patient was noted to be positive for the p53 AAb marker after treatment (Figure 10.5). This could herald the onset of recurrence despite treatment (Regele et al, 2003); although Metcalfe has refuted that serial measurement of p53 AAb can help in determining which patients recurred. Patients who were initially negative remained so during follow up whilst those that were positive also remained so. No prognostic information was gained in their study (Metcalfe et al, 2000).

For the c-myc AAb marker, two patients sero-converted to the AAb during the study (Figure 10.6). As c-myc oncogene amplification and overexpression is implicated in more aggressive tumour and poorer survival (Deming et al, 2000) (Section 3.3.3), the rise in the marker should be followed up to ascertain recurrence. Like MUC1, breast cancer treatment with anti-oestrogens can inhibit c-myc expression (Thiantanawat et al, 2003). Therefore, patients who sero-convert on treatment may signal resistance to the treatment (Venditti et al, 2002; McNeil et al; 2006).

These changes may therefore be valuable in assessing patient progress through treatment. However they may also be spurious results due to less than perfect reproducibility. With such low numbers in this group and limited follow up, we are unable to conclude accurately.

## Chapter 11

### Autoantibodies and Primary Breast Cancer

### 11.1. ALL PRIMARY BREAST CANCER

Four hundred and seven samples of 370 patients with known PBC were analysed for all 3 AAbs. These samples were subdivided into DCIS, screen-detected breast cancer and symptomatic breast cancer groups. Clinical prognosis differed for each subgroup and therefore the groups were analysed separately.

Sixty-six patients were known to have DCIS. One hundred and nineteen patients were screen detected and 187 were symptomatic patients with 222 samples available in latter group. Twenty-one symptomatic patients had multiple blood tests whilst the remainder 164 symptomatic patients had only one available sample analysed.

Two from the PBC group were male and the remaining 368 were female patients. The mean age of the PBC group was 62.9 (32 – 94) and mean age of the samples was 69.2 months (3 – 144 months). The mean follow up of the group was 56.5 months.

Table 11.1. Frequency of MUC1, p53 and c-myc AAbs (both cut-off values) in PBC cases

<b>Marker</b>	<b>+ve N (%) (Mean + 2SD)</b>	<b>+ve N (%) (Mean + 3SD)</b>
MUC1 AAb	82 (20.1%)	42 (10.3%)
p53 AAb	100 (24.5%)	85 (20.9%)
c-myc AAb	65 (15.9%)	40 (9.8%)
Panel of markers	199 (48.9%)	143 (35.1%)
Any 2 positive markers	44 (10.8%)	16 (3.9%)
All 3 positive markers	2 (0.4%)	2 (0.4%)



Fig.11.1. Distribution of PBC and subgroups for MUC1 AAbs

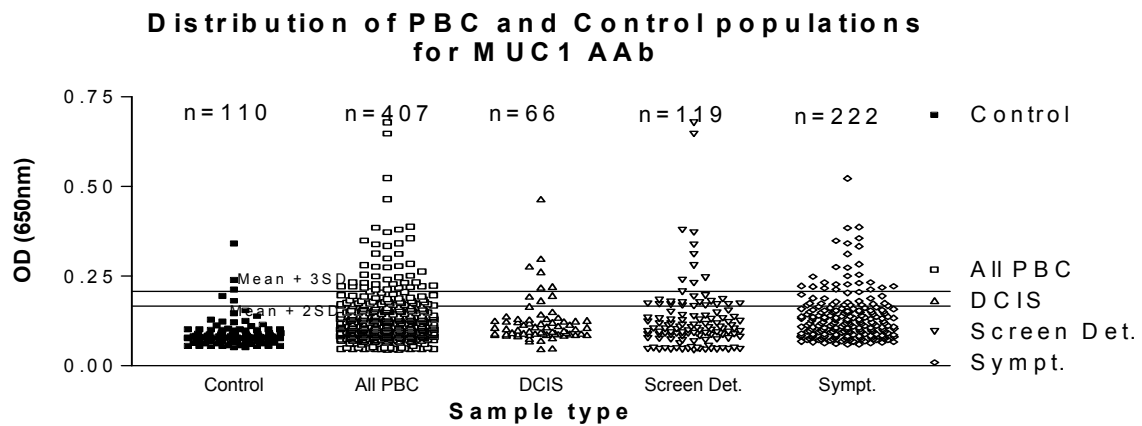


Fig.11.2. Distribution of PBC and subgroups for p53 AAbs

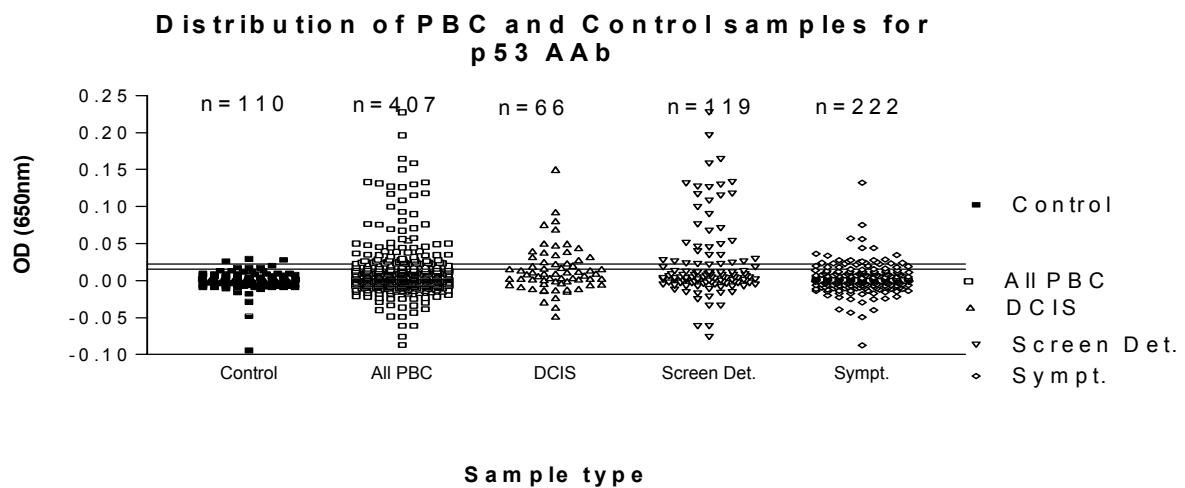
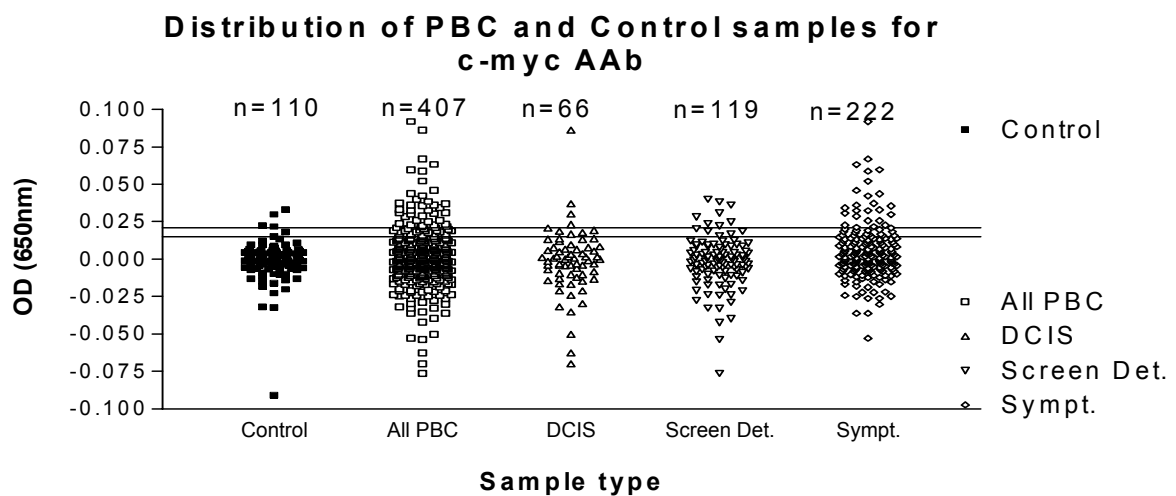


Fig.11.3. Distribution of PBC and subgroups for c-myc AAbs



For the remaining thesis only the higher cut-off value was used as it reduced false positive results without compromising sensitivity of the panel assay.

## 11.2. CARCINOMA IN-SITU

In situ carcinoma is characteristically contained within the epithelium, with the basement membrane intact, and no signs of invasion. DCIS originates from the major lactiferous ducts and tends to be a localized disease.

DCIS originates by proliferation of the ductal luminal cells, which form protrusions into the lumen (papillary DCIS). These become more coalescent, leaving a few empty, rounded spaces (cribriform DCIS). When the lumen is filled with proliferating cells, it becomes completely obliterated (solid DCIS). Central areas of these ducts undergo necrosis because of ischaemia (comedo DCIS), with secondary deposition of calcium responsible for the appearance of microcalcifications, a typical radiographic feature of this disease.

DCIS shows increasing malignant potential from the papillary to comedo forms. DCIS can be divided into 2 categories, comedo-type and non-comedo-type. DCIS may be difficult to differentiate from atypical hyperplasia (e.g. ADH and ALH), which is a benign change of the mammary gland preceding the in situ disease.

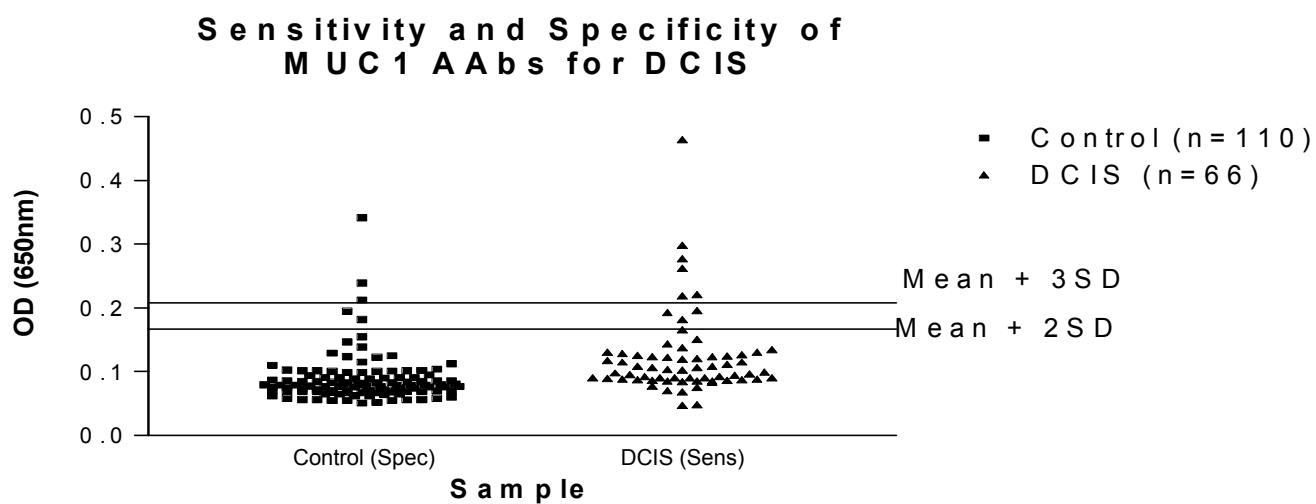
### 11.2.1. RESULTS

66 (17.8%) of the PBC samples were DCIS. The median age of the patients was 62.5 and the mean age of the samples was 21.5 months.

#### 11.2.1.1. MUC1 AUTOANTIBODY

6 (9%) were positive for the MUC1 AAb (Mean + 3SD).

Fig.11.4. Sensitivity and specificity of MUC1 AAbs for DCIS



Median OD for Control - 0.0788 (Range 0.0507 - 0.3413)

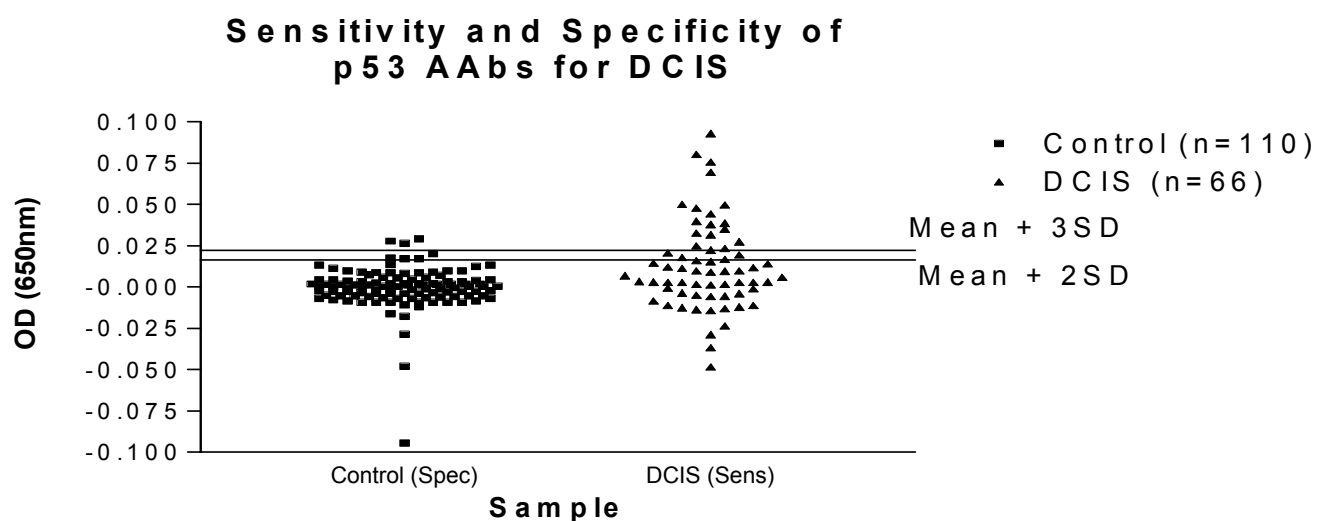
Median OD for DCIS - 0.1053 (Range 0.0473- 0.4647)

$P < 0.0001$  (Non-parametric Mann-Whitney test for significance)

### 11.2.1.2. P53 AUTOANTIBODY

20 (30.3%) were positive for the p53 AAb (Mean + 3SD)

Fig.11.5. Sensitivity and specificity of p53 AAbs for DCIS AAb



Median OD for Control: 0.0 (Range -0.0946- 0.0290)

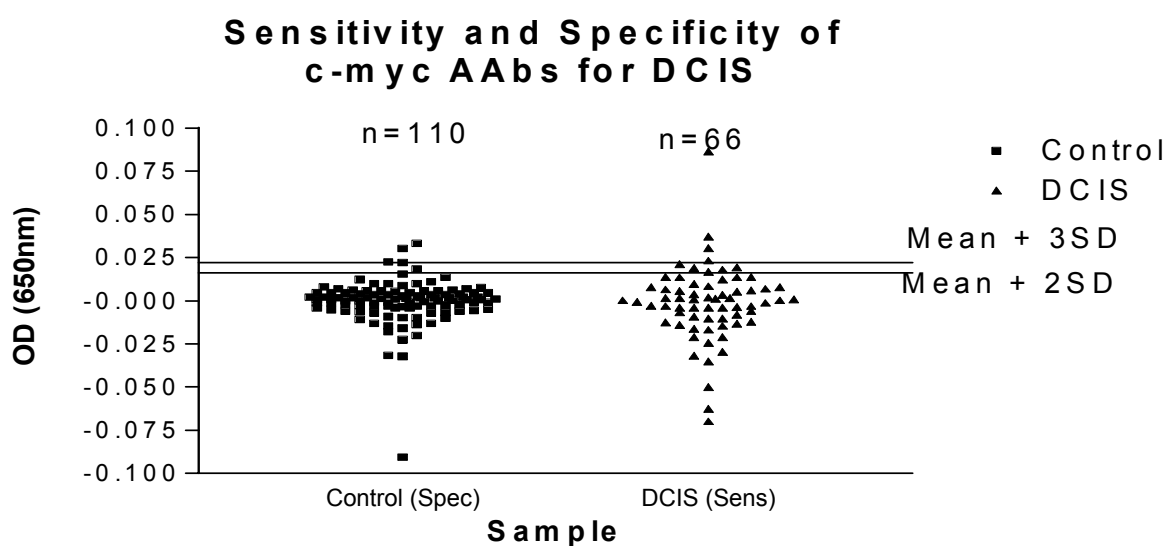
Median OD for DCIS: 0.009500 (Range -0.0487- 0.4647)

$P < 0.0001$  (Non-parametric Mann-Whitney test for significance)

### 11.2.1.3. C-MYC AUTOANTIBODY

4 (6%) were positive for the c-myc AAb (Mean + 3SD)

Fig.11.6. Sensitivity and specificity of c-myc AAbs for DCIS



Median OD for Control: 0.0003333 (Range -0.2420- 0.0330)

Median OD for DCIS: 0.0 (Range -0.0700- 0.08633)

P = 0.9574 (Non-parametric Mann-Whitney test for significance)

### 11.2.1.4. PANEL OF MARKERS

29 (43.9%) were positive for the panel of markers (Mean + 3SD)

### 11.3. DCIS CASES WITH MICROINVASION

Micro-invasion is defined as less than 1 mm invasion of tumour cells into the basement membrane. Its prognosis is still not yet understood. Only 2 cases were shown to have evidence of microinvasion. None of the 2 cases were positive for any of the tumour markers.

### 11.4. SCREEN-DETECTED CASES

The NHSBSP currently invite asymptomatic women aged between 50 and 70 for 3-yearly bilateral, 2 view mammograms. Screen-detected tumours account for almost half the diagnosed breast cancers within the given age group (Garvican and Littlejohns 1996). Screening programme is believed to provide a relative risk reduction for breast cancer mortality of between 20 to 30% compared to control women in randomised trials (WHO handbook 2002). Screening allows detection of subclinical or non-palpable breast cancer. Tumour characteristics of screen-detected cancers are more favourable than symptomatic tumours; they are smaller, more likely to be grade I or II node negative disease and have lower proliferative indices (Cortesi et al, 2006).

#### 11.4.1. RESULTS

##### 11.4.1.1. TUMOUR PATHOLOGY

Patients whose breast cancer was diagnosed by a screening mammogram were referred to as screen detected cases. There were 119 samples from 118 cases. The median age of the cases was 63.6 (range 47-80). There were no males in this group.

Table 11.2. Tumour parameters of screen-detected cases

Median Size (cm)	1.7 cm
Median Stage	1
Median Grade	2
VI +ve cases	30 (25%)
ER +ve cases	89 (75%)
Median NPI	4.18 (2.12 – 7)
• Good	48
• Moderate	51
• Poor	15
• Unknown	5

VI – Vascular invasion; ER – Oestrogen Receptor; NPI – Nottingham Prognostic Index

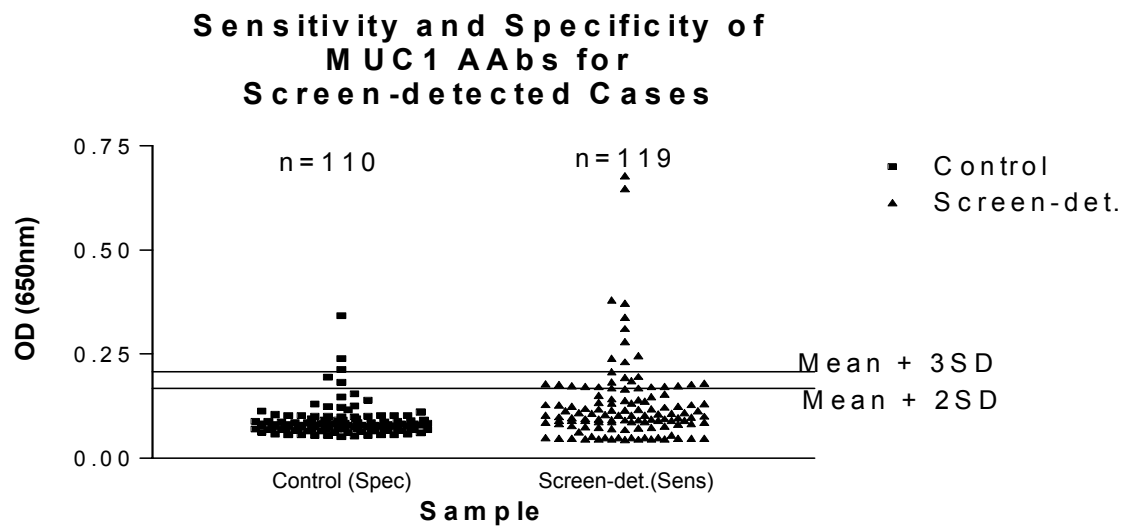
#### 11.4.1.2. TUMOUR MARKERS

All 119 samples were analysed for the presence of AAbs to MUC1, p53 and c-myc.

Table 11.3. Frequency of AAbs in screen-detected cases (Mean + 3SD)

Marker	N	% Positive
MUC1 AAb	10	8.4%
p53 AAb	37	31.1%
c-myc AAb	10	8.4%
Panel of markers	51	42.8%
Any 2 positive markers	6	5%
All 3 Positive markers	0	0%

Fig.11.7. Sensitivity and specificity of MUC1 AAbs for screen-detected cases

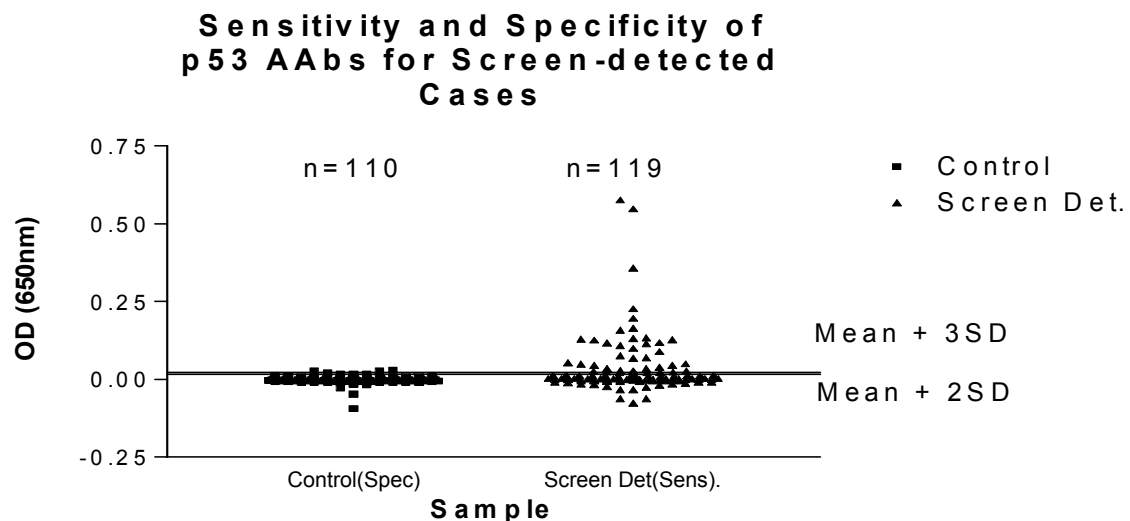


Median OD for Control - 0.0788 (Range 0.0507- 0.3413)

Median OD for Screen-detected - 0.1037 (Range 0.0447- 0.6787)

$P < 0.0001$  (Non-parametric Mann-Whitney test for significance)

Fig.11.8. Sensitivity and specificity of p53 AAbs for screen-detected cases



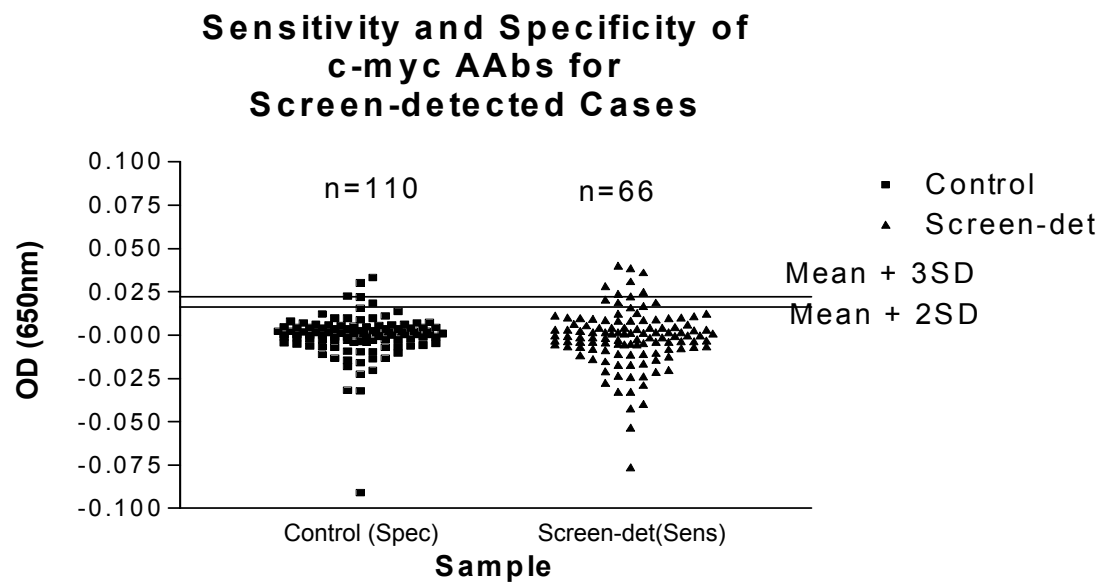
Median OD for Control: 0.0 (Range -0.0947- 0.0290)

Median OD for Screen-detected: 0.0056 (Range -0.0763- 0.5770)

$P < 0.0001$  (Non-parametric Mann-Whitney test for significance)



Fig.11.9. Sensitivity and specificity of p53 AAbs for screen-detected cases



Median OD for Control: 0.0003 (Range -0.2420- 0.0330)

Median OD for Screen-detected: -0.0007 (Range -0.4843- 0.2667)

P = 0.794 (Non-parametric Mann-Whitney test for significance)

## 11.5. SYMPTOMATIC BREAST CANCER

Symptomatic breast cancers account for the majority of diagnosed breast cancer. Usual presentation is of a painless lump within the breast tissue. Other presentations include deformity, nipple retraction, nipple discharge, skin puckering and pain. Diagnosis is based on triple assessment i.e. clinical, radiological and histological assessment. In comparison to screen-detected tumours, symptomatic breast cancers are generally larger, more like to be stage III and of a higher grade (Cowan et al, 1997). These characteristics worsen the prognosis with reduced survival and increased recurrence rates compared to screen-detected cases. However the actual overall survival is still good at 85% at 5 years (Yassin et al, 2003). This reduces with time to 52% at 20 years according to the American Cancer Society. The survival rates will differ in the various prognostic groups.

### 11.5.1. RESULTS

#### 11.5.1.1. TUMOUR PATHOLOGY

Patients who presented with a palpable lump or any other symptoms resulting in the diagnosis of breast cancer are referred to as symptomatic cases. 222 samples from 187 cases were analysed for the presence of the AAbs to MUC1, p53 and c-myc.

186 were female and 1 male. The mean age of this group of patients was 65.5 (range 32-94).

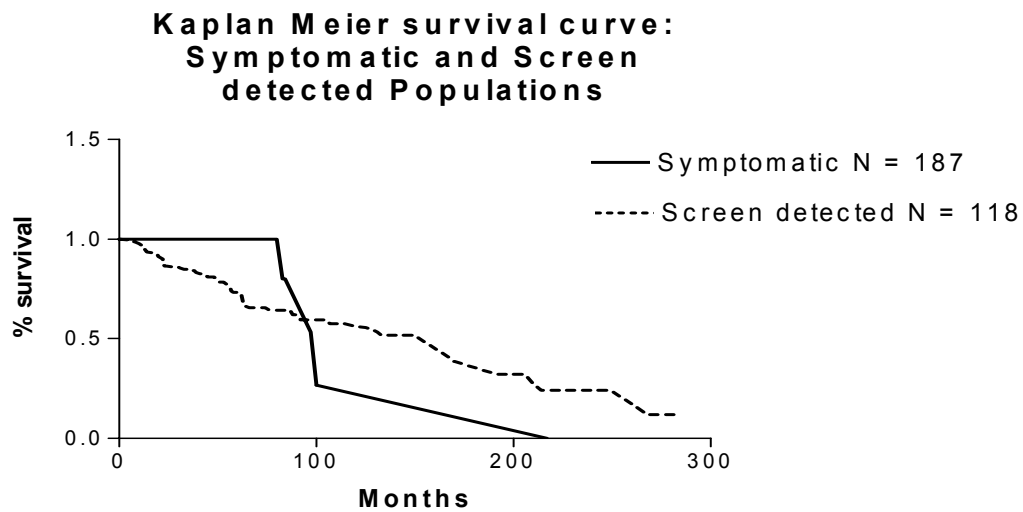
Table 11.4. Tumour pathology of symptomatic in comparison to screen-detected cases

	<b>Screen-Detected</b> <b>N=118</b>	<b>Symptomatic</b> <b>N=187</b>	<b>P value</b>
Median Size (mm)	1.7	2.3	<0.0001
Median Stage	1	1	0.0240
Median Grade	2	3	0.0025
VI +ve cases	42 (36.8%)	83 (56%)	0.0020
ER +ve cases	89 (82%)	72 (65.4%)	0.0044
Median NPI (range)	4.18 (2.12 – 7)	4.40 (2.18 – 7.8)	0.0006
• Good	50	44	P<0.0001
• Moderate	49	81	
• Poor	15	36	
• Unknown	4	61	

Median values compared by Mann-Whitney non-parametric test for significance

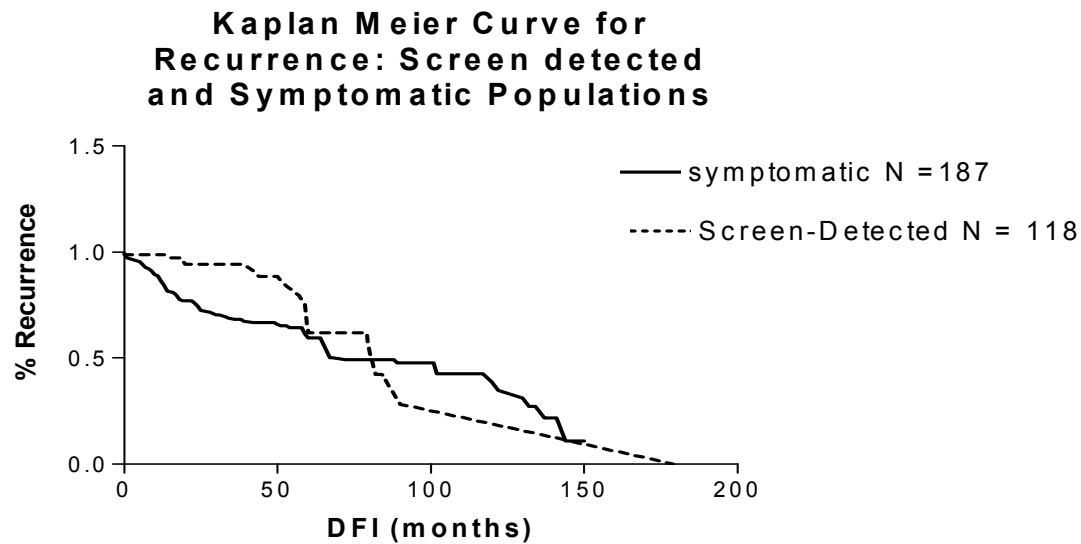
VI and ER positive cases compared by Fishers exact test for significance

Fig.11.10. The following survival curve shows survival comparison between the screen-detected and symptomatic cases



Logrank test for significance:  $p=0.0128$  (significant)

Fig.11.11. The following Kaplan-Meier curve compares any form of recurrence in the screen-detected and symptomatic cases



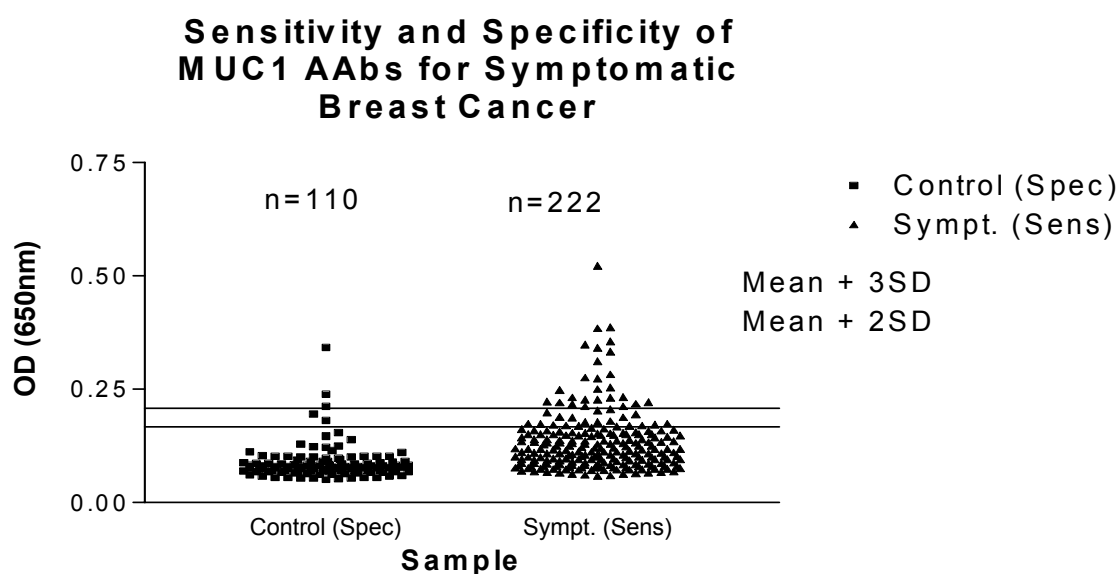
Logrank test for significance:  $p=0.016$  (significant)

### 11.5.1.2. TUMOUR MARKERS

Table 11.5. Frequency of MUC1, p53 and c-myc AAbs in symptomatic cases (Mean + 3SD)

Marker	Symptomatic (N=222)	(%)
MUC1 AAb	26	11.7%
p53 AAb	28	12.6%
c-myc AAb	26	11.7%
Panel of markers	66	29.7%
Any 2 positive markers	13	5.8%
All 3 Positive markers	1	0.4%

Fig.11.12. Sensitivity and specificity of MUC1 AAbs for symptomatic breast cancer

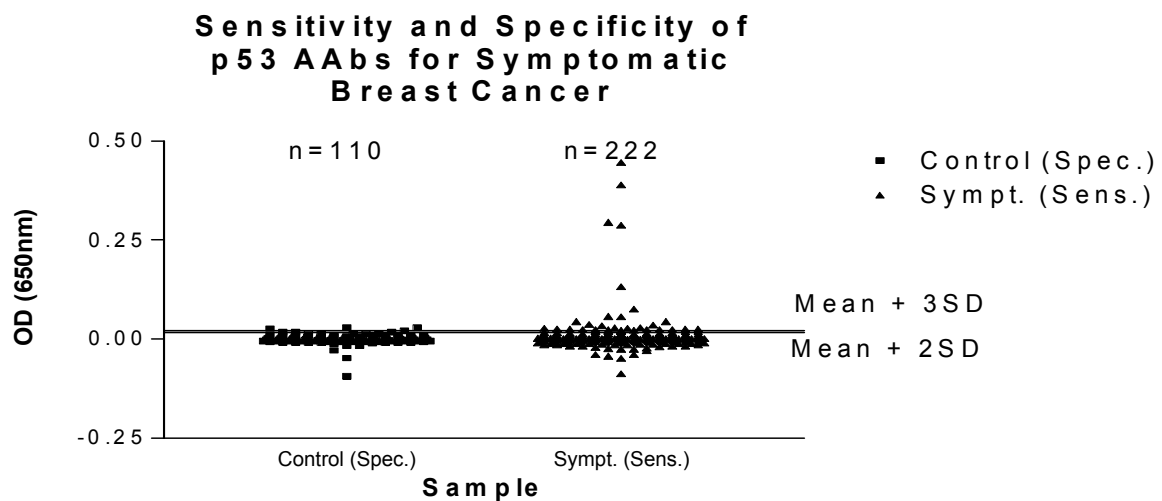


Median OD (650nm) for Control - 0.0788 (Range 0.0507- 0.3413)

Median OD (650nm) for Symptomatic - 0.1190 (Range 0.0600- 0.5227)

P < 0.0001 (Non-parametric Mann-Whitney test for significance)

Fig.11.13. Sensitivity and specificity of p53 AAbs for symptomatic breast cancer

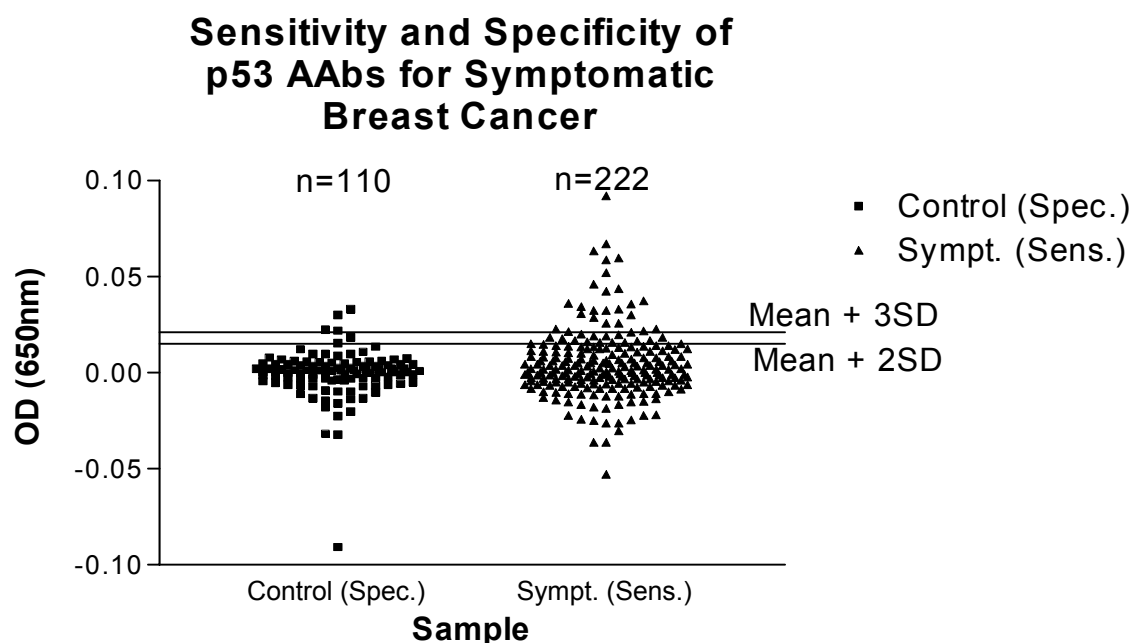


Median OD (650nm) for Control: 0.0 (Range -0.09467- 0.0290)

Median OD (650nm) for Symptomatic: 0.001333 (Range -0.08733- 0.4453)

P = 0.1397 (Non-parametric Mann-Whitney test for significance)

Fig.11.14. Sensitivity and specificity of c-myc AAbs for symptomatic breast cancer



Median OD (650nm) for Control: 0.0003 (Range -0.2420- 0.0330)

Median OD (650nm) for Symptomatic: 0.0016 (Range -0.0530- 0.1150)

P = 0.0190 (Non-parametric Mann-Whitney test for significance)

## 11.6. DISCUSSION

PBC can be divided into pre-invasive (DCIS) and invasive cancers. Both groups are distinct in their natural history, treatment and prognosis. Furthermore for purpose of the study, we also subdivided the invasive tumours into screen-detected and symptomatic cancers. Although the two are biologically similar tumours (Cowan et al, 1997), their treatment and prognosis differ. This is demonstrated in our data (Table 11.4, Figures 11.10 and 11.11).

Results of our DCIS sample at the Mean + 3SD cut-off value had shown positive markers for all three AAbs, with p53 AAb the most prevalent at 30.3% (Sections 11.2.1.1 to 11.2.1.4). Prevalence for both MUC1 and c-myc AAbs were 9% and 6% respectively. A panel of all 3 markers gave a prevalence of 43.9%. Both MUC1 and p53 AAbs in DCIS were significantly higher than in Control cases (Figures 11.4 and 11.5). c-myc AAb frequencies in the two groups were similar (Figure 11.6).

The panel for DCIS was significantly more sensitive than for the Control and At-risk populations (Tables Appendix 1 and Appendix 2). DCIS is pre-invasive disease and an immune response to antigens may be due to presence of infiltrating B lymphocytes in DCIS stroma (Shimokawara et al, 1982). These B cells are responsible for AAb production.

MUC1 antigen is detected in both membranous and cytoplasmic compartments of virtually all breast epithelium in pure DCIS tissue (Diaz et al, 2001). However in only 50% of cases is it possible to detect immune complexes of MUC1 in the sera of these DCIS cases (von Mensdorff-Pouilly et al, 1996).

Our data indicated that the p53 AAb was the most prevalent amongst the different markers in the DCIS population (30.3%). This result further implicates p53 abnormality as an early event in carcinogenesis (Shi et al, 1999; Campbell et al, 1993). When DCIS is compared to invasive cancer, only the p53 AAb sensitivity was significantly higher in the former group (Figure Appendix 7). This difference may highlight tumour biology in early evolution of breast cancer where p53 mutation is pivotal (Singh et al, 1993).

c-myc oncogene amplification is associated with more advanced, poor prognostic breast cancer (Berns et al, 1992, Deming et al, 2000). It is therefore not expected to be prevalent in great quantities in pre-invasive DCIS. Our data suggested only 6% c-myc AAb prevalence in the sera (section 11.2.1.3). This corresponded well with previous data from the Unit from Cheung who had detected 4.2% using the same cut-off value as ours. More recent work from the unit (Chapman et al, 2007) further established a low prevalence of c-myc in DCIS (8%). The latter work used a slightly different assay to the current authors with the cut-off value at Mean +2SD. Our database only had two pathologically determined DCIS with micro invasion. It is therefore not possible to draw any conclusions from such small numbers.

Screen detected breast tumours are detected by mammographic surveillance under the NHSBSP. They are limited to women aged 50 to 70 although women beyond 70 can request further screening if so requests. Cancers noted via the NHSBSP tend to be more in situ and if invasive, to be smaller, of lower grade and to have invaded vessels, peri-neural spaces and lymph nodes less frequently (Cortesi et al, 2006; Cowan et al, 1997). However IHC has failed to detect any difference in the staining for p53, or other antigens considered related to tumour behaviour in screen-detected cases compared to symptomatic breast cancer (Cowan et al, 1997). This implies a tumour that is essentially biologically similar to symptomatic breast



cancer. There are as yet no data on c-myc and MUC1 antigen prevalence in screen-detected tissues.

Data on serum p53 AAb in PBC cases varies between 5% (Angelopoulou et al, 1994) to 48% (Willsher et al, 1996) according to the study, method of detection and population size. Prevalence amongst screen-detected cases in our study was 31.1% (Table 11.3). This was much higher than some of the previous studies but similar to others looking at breast cancers as a whole (Table 3.2). This may be a consequence of the technique, numbers of cases analysed and cut-off value used. We were also analysing screen-detected cases, a group that is generally not examined specifically in other studies. This population difference may account for some of the differences noted in the literature.

As already recognised our results on symptomatic patients confirmed that these tumours were larger, higher grade, more likely to invade vessels and are generally of poorer prognosis when compared to screen-detected cases (Table 11.4). Furthermore the former is less likely to be ER positive compared to the latter, again another feature of poorer prognosis. This translates well into a shorter OS in the symptomatic group as noted by the survival curve (Figure 11.10). Furthermore, these factors also increased the likelihood of recurrence with a shorter DFI (Figure 11.11).

MUC1 AAb sensitivity in our assay for symptomatic patients was 11.7% (Table 11.5). This was similar to our previous data on screen-detected, DCIS and even healthy at-risk patients (Table 14.1). Published data have reported that up to one third of breast and ovarian cancer patients may have circulating antibodies reactive to MUC1 (von Mensdorff-Pouilly, 1998). Our method did not measure circulating immune complexes. CICs have been reported to

account for half of all AAbs produced during the humoral immune response. Furthermore, the discrepancy may also have arisen because our data for the MUC1 AAb as previously highlighted was based on an antigen purified from the pleural effusion of a single advanced breast cancer patient thus potentially limiting the amount and type of antigen attained in comparison to antigens purified from a pooled group of patients.

We demonstrated 12.6% of symptomatic patients had anti-p53 antibodies in their serum at the time of detection (Table 11.5). This was considerably lower compared to both screen-detected and DCIS cases. It would suggest that an immune response to p53 is an early event in the tumour cascade. The results for anti-p53 antibody in our symptomatic cases were also lower than those detected in our unit by Cheung (28%) and Chapman (24%). However both previous works had not subdivided the PBC cases into screen-detected and symptomatic cases, which may account for some of the discrepancy. Another comparable study from USA had noted p53 AAbs in serum in only 7.8% of breast cancer patients (Meghiorino et al, 2005). The methodology of this particular study was very similar to ours as their cut-off was set at the higher mean + 3SD level. However their breast cancer population number was only 105. General review of the literature has shown that there are other studies whose data for p53 AAb frequency in breast cancer patients is consistent with our own i.e. Angelopoulou et al (5%) (Angelopoulou et al, 1994) Dalifard et al (7%) (Dalifard et al, 1999), Metcalfe et al (15%) (Metcalfe et al, 2000) and Balogh et al (16.36%) (Balogh et al, 2006). Other authors have detected greater frequencies compared to our own data for p53 AAb in breast cancer i.e. Willsher (48%) (Willsher et al, 1996), Mudenda (26%) (Mudenda et al, 1994) and Green (25.6%) (Green et al, 1994). All of the above authors have used ELISA as the detection methodology.

The prevalence of c-myc AAb in the serum of symptomatic patients was 11.7% (Table 27). This was significantly lower than that previously published from our unit by Cheung (31%) (Cheung 2001) but consistent with more recent data from the Unit by Chapman (13%) (Chapman 2007) as well as the study from Megliorino (18.8%) (Megliorino et al, 2005). The discrepancy may be accounted by the difference in assay protocol, number of patients used and subdivision of the PBC population into symptomatic and screen-detected cases.

The overall prevalence of any one marker within the panel was 29.7% (Table 11.5) in symptomatic group and 35.1% in the overall PBC group. This was lower than previously noted by Cheung (74%) and Chapman (64%). Panel sensitivity for Megliorino was 26.6%, a figure similar to ours (Megliorino et al, 2005). Their cut-off was set at the higher Mean + 3SD, comparable to ours and unlike the other two set of data. Megliorino's panel differed from ours as survivin was substituted for MUC1.

Although the current data as presented in this thesis was limited by the low sensitivity for some of the markers, the panel showed promising results in the PBC group. Combination of different tumour associated antigens within a panel generally increases the detection rate for the specific cancer with different cancers requiring different panels (Zhang 2004 and 2007). With specificity close to 85%, the findings in this study raises the future possibility that with further development of the assays for AAbs both individually and as a panel, serum AAbs as tumour markers could potentially be used as an adjunct to imaging in the detection of primary breast cancers.

## Chapter 12

### Autoantibodies and Prognosis

## 12.1. FOLLOW UP OF PBC

All primary breast cancer cases were followed up to end of study period and data on survival and recurrence were attained from pathology reports and in-house database where available. Types of recurrence i.e. loco-regional or metastatic were identified. As screen-detected tumours were more favourable, they were categorised separately from symptomatic tumours and data presented in this chapter therefore depicted PBC as DCIS, screen-detected and symptomatic.

## 12.2. DCIS

Table 12.1. Correlation of positive AAbs and grade of DCIS

<b>Marker</b>	<b>Unknown (15)</b>	<b>Low grade (5)</b>	<b>Intermediate (16)</b>	<b>High (30)</b>	<b>Fishers exact test</b>	<b>Significance</b>
MUC1 AAb +ve	3	0	2	4	0.387	ns
p53 AAb +ve	5	1	8	10	0.659	ns
c-myc AAb +ve	2	0	3	4	0.683	ns
Panel of markers	9	1	9	17	0.636	ns
Any 2 +ve markers	1	0	2	1	0.596	ns
All 3 +ve markers	0	0	1	0	0.366	ns

### 12.2.1. RECURRENCE

As the mean follow up of DCIS cases was only 40.2 months (Range 11- 93months) and there were so few in each group, any recurrence analysis would be of little relevance and therefore not performed.

### 12.3. SCREEN-DETECTED

#### 12.3.1. MUC1 AUTOANTIBODY

Table 12.2. Tumour parameters for +ve and -ve MUC1 AAb cases for screen-detected population

Parameters	+ve MUC1 AAb (N = 10)	-ve MUC1 AAb (N = 109)	P	Significant
Median size (cm)	1.35cm	1.7cm	0.455	ns
Median Stage	1	1	0.680	ns
Median Grade	2	2	0.099	ns
VI +ve	2/10	36/109	0.324	ns
ER +ve cases	7/10	89/109	1	ns
Median NPI	3.13 (2.12 – 5.7)	4.20 (2.16 – 7)	0.176	ns

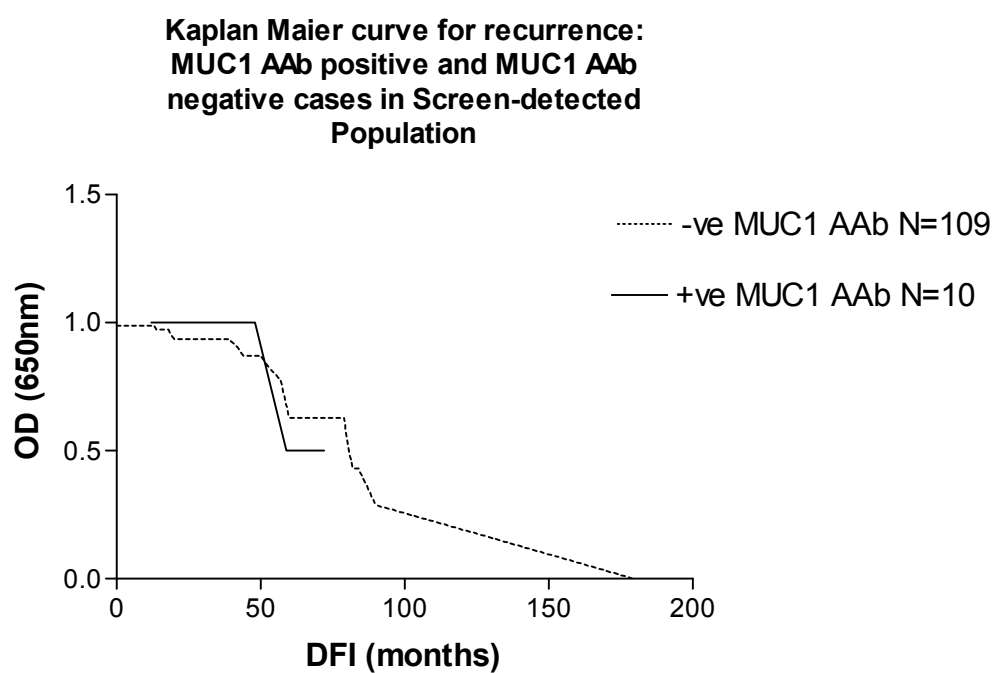
Median values compared using Mann-Whitney non-parametric test for significance

VI and ER +ve cases compared between the two groups by chi-squared

### 12.3.1.1. RECURRENCE / SURVIVAL

The mean follow up of the screen-detected cases was 40 months. The mean follow up of positive and negative MUC1 AAb groups were 48 and 39 months respectively.

Fig.12.1. Recurrence between +ve and -ve MUC1 AAb cases for screen-detected population



Logrank test for significance:  $p = 0.591$  (non significant)

Logrank test for significance for survival difference between MUC1 AAb positive and negative cases was  $p = 0.657$  (ns).

### 12.3.2. P53 AUTOANTIBODY

Table 12.3. Tumour parameters for +ve and -ve p53 AAb cases for screen-detected population

Parameters	+ve p53 AAb (N = 37)	-ve p53 AAb (N = 82)	P	Significant
Median size (cm)	1.70 cm	1.70 cm	0.497	ns
Median Stage	1	1	0.22	ns
Median Grade	2	2	0.049	Significant
VI +ve	20/37	22/82	0.008	Significant
ER +ve cases	28/37	61/82	1	ns
Median NPI	4.4 (2.24 – 6.9)	3.46 (2.12 – 7)	0.014	Significant

Median values compared using Mann-Whitney non-parametric test for significance

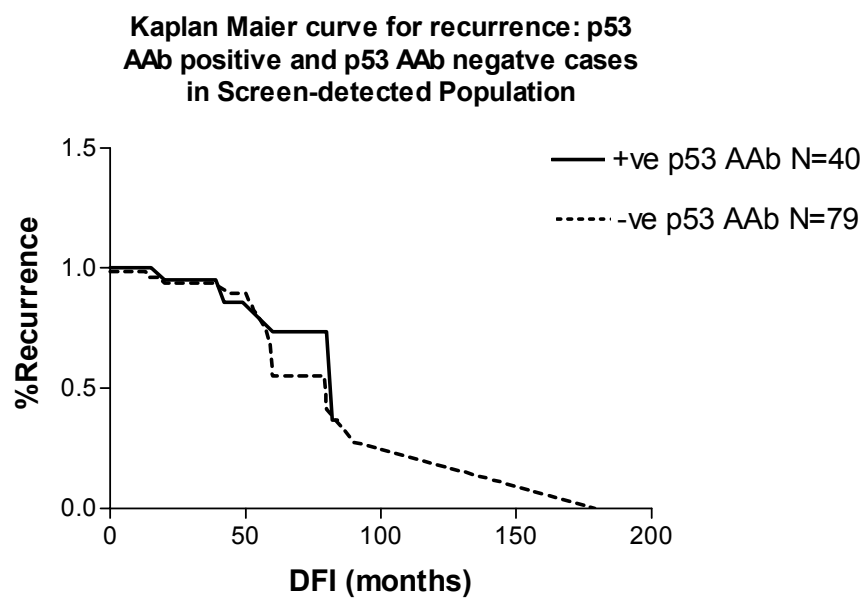
VI and ER positive cases compared between the two groups by chi-squared



### 12.3.2.1. RECURRENCE / SURVIVAL

The mean follow up of positive and negative p53 AAb groups were 38.5 and 34.6 months respectively.

Fig.12.2. Recurrence between +ve and --ve p53 AAb cases for screen-detected population



Logrank test for significance:  $p = 0.5253$  (non significant)

Logrank test for significance for OS difference between p53 AAb positive and negative cases was  $p = 0.760$  (ns)

### 12.3.3. C-MYC AUTOANTIBODY

Table 12.4. Tumour parameters for +ve and -ve c-myc AAb cases for screen-detected population

Parameters	+ve c-myc AAb (N = 10)	-ve c-myc AAb (N = 109)	P	Significant
Median size (cm)	1.35 cm	1.70 cm	0.146	ns
Median Stage	1	1	0.683	ns
Median Grade	2	2	0.574	ns
VI +ve	3/10	39/109	0.378	ns
ER +ve cases	8/10	82/109	1	ns
Median NPI	3.18 (2.24 – 6.42)	4.2 (2.12 – 7)	0.4525	ns

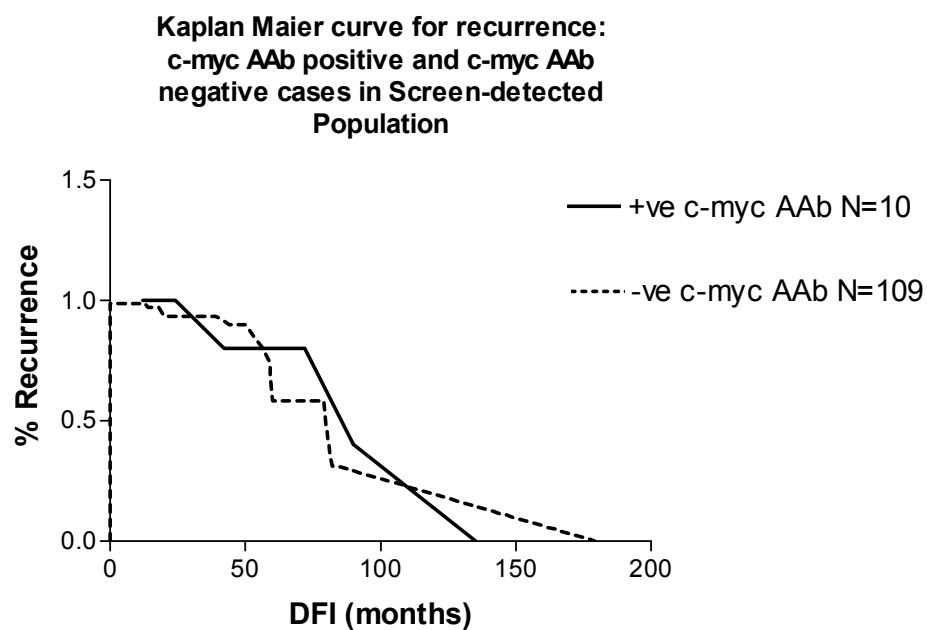
Median values compared using Mann-Whitney non-parametric test for significance

VI and ER positive cases compared between the two groups by chi-squared

### 12.3.3.1. RECURRENCE / SURVIVAL

The mean follow up of positive and negative c-myc groups were 35 and 35 months respectively.

Fig.12.3. Recurrence between +ve and -ve c-myc AAb cases for screen-detected population



Logrank test for significance:  $p = 0.703$  (non significant)

Logrank test for significance for OS difference between c-myc AAb positive and negative cases was  $p = 0.763$  (ns)

#### 12.3.4. PANEL OF MARKERS

Table 12.5. Tumour parameters for +ve and -ve panel cases for screen-detected population

Parameters	+ve panel (N = 51)	-ve panel (N = 68)	P	Significant
Median size (cm)	1.65 cm	1.7 cm	0.819	ns
Median Stage	1	1	0.1224	ns
Median Grade	2	2	0.205	ns
VI +ve	18 (34%)	20 (30.3%)	0.266	ns
ER +ve cases	37 (72%)	52 (76%)	0.666	ns
Median NPI	4.32 (2.12 – 6.9)	3.59 (2.16 – 7)	0.112	ns

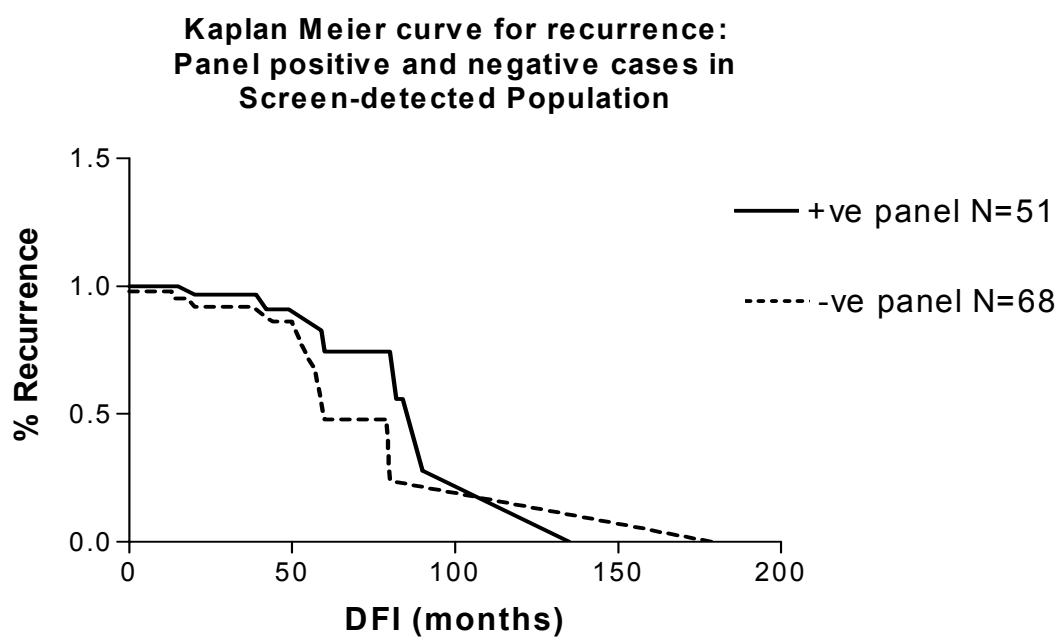
Median values compared using Mann-Whitney non-parametric test for significance

VI and ER positive cases compared between the two groups by chi-squared

#### 12.3.4.1. RECURRENCE / SURVIVAL

The mean follow up of positive and negative panel groups were 37.1 and 34.6 months respectively.

Fig.12.4. Recurrence between +ve and -ve panel for screen-detected population



Logrank test for significance:  $p = 0.261$  (non significant)

Logrank test for significance for OS difference between panel positive and negative cases was  $p = 0.949$  (non-significant)

## 12.4. SYMPTOMATIC

The mean follow up of the symptomatic group was 79.3 (8-281) months.

### 12.4.1. MUC1 AUTOANTIBODY

Table 12.6. Tumour parameters for +ve and -ve MUC1 AAb cases for symptomatic population

Parameters	+ve MUC1 AAb (N=26)	-ve MUC1 AAb (N=196)	P	Significant
Median size (cm)	2.15	2.30	0.319	ns
Median Stage	1	1	0.654	ns
Median Grade	2	3	0.125	ns
VI +ve	10/26 (38%)	75/196 (38.1%)	0.369	ns
ER +ve cases	15 (57.7%)	92 (46.9%)	0.531	ns
Median NPI (range)	4.24 (2.18 – 6.56)	4.32 (2.2 – 7.8)	0.760	ns

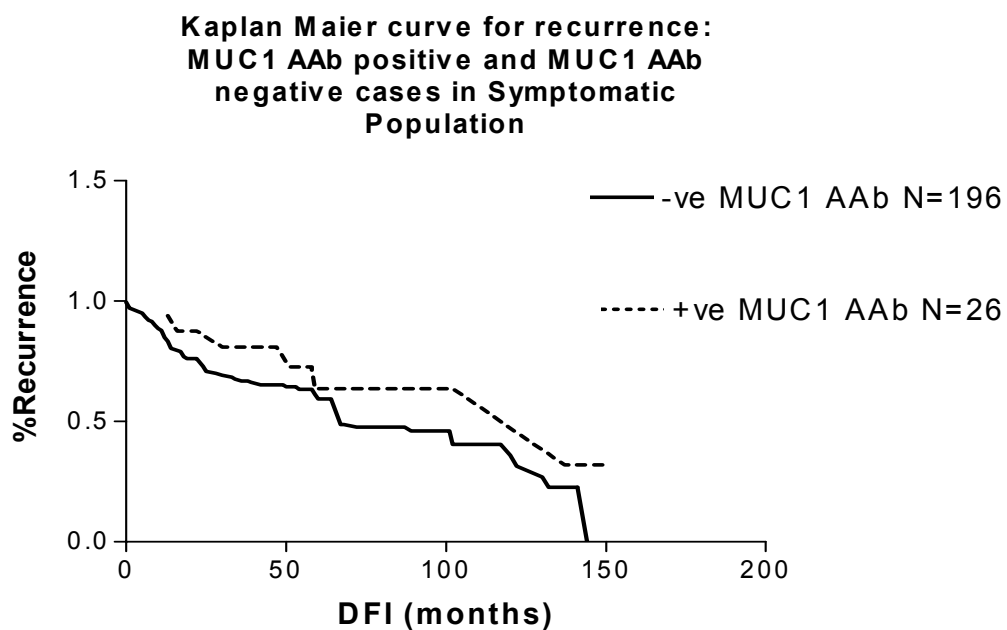
Median values compared using Mann-Whitney non-parametric test for significance

VI and ER positive cases compared between the two groups by chi-squared

#### 12.4.1.1. SURVIVAL / RECURRENCE

The mean follow up of positive and negative MUC1 AAb cases were 79.9 and 79.2 months respectively.

Fig.12.5. Recurrence between +ve and -ve MUC1 AAb cases for symptomatic population



Logrank test for significance  $p = 0.1733$  (Non Significant).

Logrank test for significance for OS difference between MUC1 AAb positive and negative cases was  $p = 0.760$  (non-significant)

### 12.4.2. P53 AUTOANTIBODY

Table 12.7. Tumour parameters for +ve and -ve p53 cases for symptomatic population

Parameters	+ve p53 AAb (N=28)	-ve p53 AAb (N=194)	P	Significant
Median size (cm)	2.80	2.20	0.542	ns
Median Stage	2	2	0.574	ns
Median Grade	3	3	0.376	ns
VI +ve	13 (46.4%)	94 (48.4%)	0.513	ns
ER +ve cases	7 (25%)	65 (33%)	0.085	ns
Median NPI (range)	4.58 (2.18 – 7.2)	4.07 (2.22 – 7.8)	0.342	ns

Median values compared using Mann-Whitney non-parametric test for significance

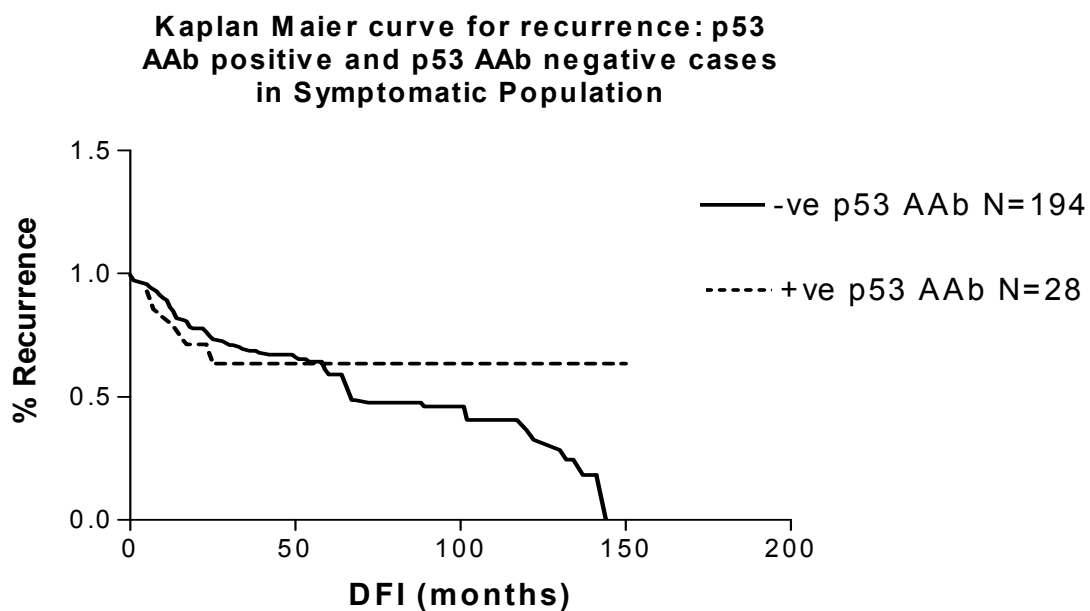
VI and ER positive cases compared between the two groups by chi-squared



#### 12.4.2.1. RECURRENCE / SURVIVAL

The mean follow up of positive and negative p53 AAb groups were 67.5 and 80.7 months respectively.

Fig.12.6. Recurrence between +ve and --ve p53 AAb cases for symptomatic population



Logrank test for significance  $p = 0.3164$  (Non Significant).

Logrank test for significance for OS difference between p53 AAb positive and negative cases was  $p = 0.906$  (non-significant)

### 12.4.3. C-MYC AUTOANTIBODY

Table 12.8. Tumour parameters for +ve and -ve c-myc AAb cases for symptomatic population

Parameters	+ve c-myc AAb (N=26)	-ve c-myc AAb (N=196)	P	Significant
Median size (cm)	3.0	2.20	0.122	ns
Median Stage	1	1	0.792	ns
Median Grade	3	3	0.719	ns
VI +ve	12 (46%)	72 (36.7%)	0.811	ns
ER +ve cases	10 (38.4%)	101 (51.5%)	0.132	ns
Median NPI (range)	4.6 (3.28 – 7.0)	4.3 (2.18 – 7.8)	0.436	ns

Median values compared using Mann-Whitney non-parametric test for significance

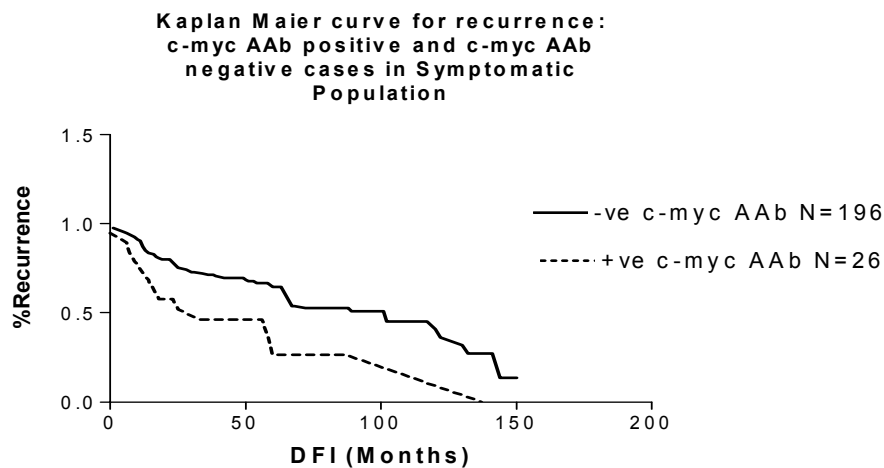
VI and ER positive cases compared between the two groups by chi-squared

### 12.4.3.1. RECURRENCE / SURVIVAL

The mean follow up of positive and negative c-myc AAb groups were 74.3 and 79.4 months respectively.

Median DFI for -ve c-myc AAb = 80 months: Median DFI for +ve c-myc AAb = 18 months

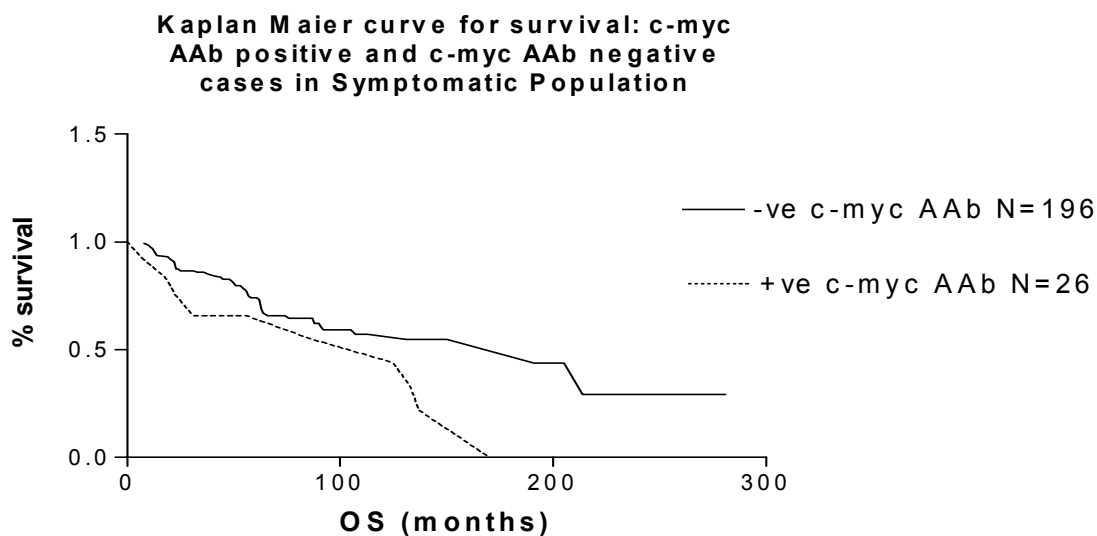
Fig.12.7. Recurrence between +ve and -ve c-myc AAb cases for symptomatic population



Logrank test for significance  $p = 0.0115$  (Significant).

Median OS for c-myc AAb negative = 191 months: Median OS for c-myc AAb positive = 125 months

Fig.12.8. OS between +ve and -ve c-myc AAb cases in symptomatic group



Logrank test for significance  $p = 0.0248$  (Significant).

#### 12.4.4. PANEL OF MARKERS

Table 12.9. Tumour parameters for +ve and -ve panel cases for symptomatic population

Parameters	+ve panel (N=66)	-ve panel (N=156)	P	Significant
Median Size(cm)	2.8	2.0	0.3194	ns
Median Stage	1	1	0.520	ns
Median Grade	3	3	0.841	ns
VI +ve	35 (53%)	72 (46.9%)	0.861	ns
ER +ve cases	30 (45.4%)	110 (70.5%)	0.057	ns
Median NPI (range)	4.36 (2.18–7.2)	4.28 (2.22 – 7.8)	0.252	ns

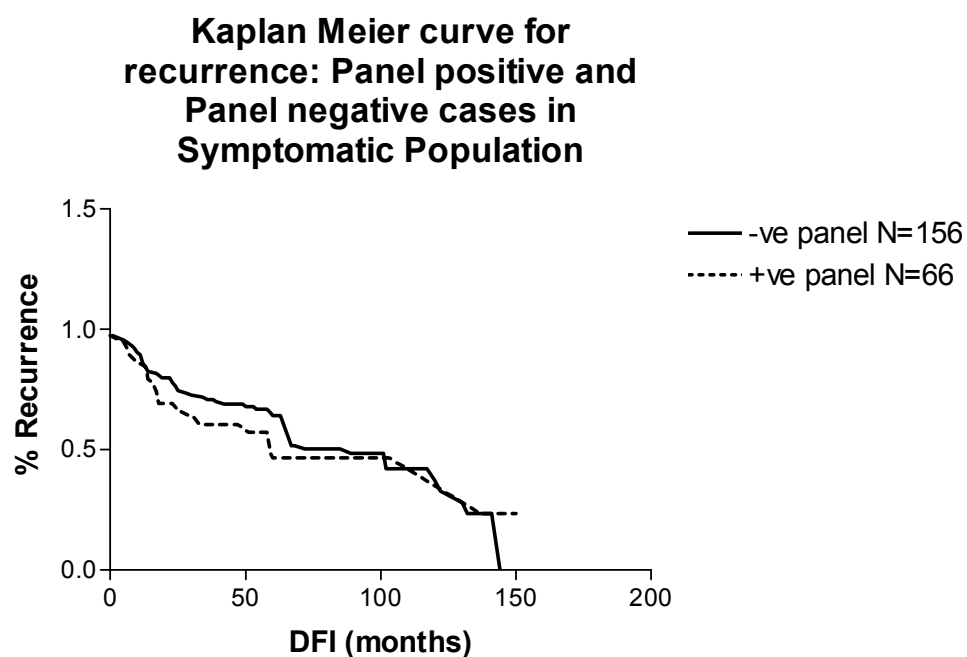
Median values compared using Mann-Whitney non-parametric test for significance

VI and ER positive cases compared between the two groups by chi-squared

#### 12.4.4.1. RECURRENCE / SURVIVAL

The mean follow up of positive and negative panel groups were 73.1 and 81.3 months respectively.

Fig.12.9. Recurrence between +ve and -ve panel for symptomatic population



Logrank test for significance  $p = 0.6039$  (Non Significant).

Logrank test for significance for OS difference between panel positive and negative cases was  $p = 0.4912$  (Non Significant).

## 12.5. DISCUSSION

The relevance to some tumours showing a humoral response may be in its prognosis, as tumours with CIC have higher OS (von Mensdorff-Pouilly et al, 1996). There appeared to be no difference in expression of MUC1 AAb and grade of DCIS (Table 12.1). Furthermore our data did not appear to provide any evidence regarding any of the other markers detected and grade of DCIS (Table 12.1). This was consistent finding from previous data at our institution (Cheung 2001) and more recent data from Chapman (Chapman et al, 2007) who also failed to detect any concordance between AAb detection and grade of DCIS. However the cut-off value used in both data was the lower Mean + 2SD and the population sizes were small in both studies (24 and 40 respectively).

Although our data did not suggest p53 AAb correlated with tumour grade in DCIS, Yang had shown that tissue p53 antigen did indicate higher grade in this group (Yang et al, 2003). A higher recurrence rate in this group of pre-invasive breast cancer was also noted by Hieken (Hieken et al 2007). We did not calculate recurrence for DCIS as population size and follow up was too small.

Data from Aulmann showed c-myc antigen to be present in 20% of DCIS tissue (Aulmann et al, 2002). They correlated c-myc oncogene amplification in DCIS with larger tumours and higher proliferative activity. Our data did not show a statistical significance between the different grade of DCIS and c-myc AAb prevalence; 4 of our 7 positive c-myc AAb cases (57%) were high grade DCIS (Table 12.1). Aulmann further noted 89% of his cases were either grade 2 or 3, results that mimic ours (7 out of 7 are either high or intermediate grade) although 2 unknown grades may obscure the true result.

In both screen-detected and symptomatic PBC cases the panel of markers failed to indicate prognosis. However in screen-detected group p53 AAb detection conferred poorer prognostic parameters such as grade and VI, with an overall higher NPI (Table 12.3). This did not however translate into overall lower DFI or OS, according to the Kaplan Meier curve (Figure 12.2) and Logrank test for significance for survival.

Comparison of prognostic data with previous work (Cheung, 2001) also noted no significance of the panel or any of the individual markers in loco-regional recurrence for PBC cases. For distant recurrence, Cheung had demonstrated that the presence of MUC1 and p53 AAbs though not the panel, indicated a shorter time to distant metastasis and poorer survival. This was contradictory to our own data where no prognosis was conferred from the detection of AAbs to MUC1 and p53 in PBC cases. The differences in outcome between the two studies from the same unit may be explained by the differences in assays used (basis of the present thesis) and time to follow up (79 months in current work and 51 months in previous work).

Published data on p53 AAb may indicate a poorer prognosis with a shorter DFI in breast cancer (Sangrajrang et al, 2003; Volkmann et al, 2002; Lenner 1999). However data from Huobner (Huobner 1996), Regidor (Regidor 1996) Wilsher (Wilsher 1996) Dalliford (Dalliford 1999) and Metcalfe (Metcalfe et al, 2000) contradict the above studies, as they show that no prognostic information is gained from detecting p53 AAbs in the sera of breast cancer patients (Table 3.2). p53 AAb status has also been reported to signify histological type of breast tumour (Sangrajrang et al, 2003; Dalifard et al, 1999) and site of likely metastasis i.e. more lung than bone (Crawford et al, 1982). Discrepancies on prognostic information may be due to the differing methods used in the studies to detect the p53 AAb, such as an immunoluminometric method ((Dalifard et al, 1999) or ELISA (Willsher et al, 1996;

Metcalfe et al, 2000) as well as multiple possible epitopes available on a mutant p53 antigen and hence multiple anti-p53 antibodies available that can be detected by various methods. Discrepancies in follow-up length, sample size, cut-off values and assay sensitivity may also result in varying results. From our data we conclude that a humoral immune response to MUC1 and p53 did not appear to impact survival in symptomatic breast cancers.

Although our results failed to detect any discernible difference in tumour pathology between those with c-myc AAbs and those without in both screen-detected and symptomatic group (Tables 12.4 and 12.8), the c-myc AAb positive population in the symptomatic group had a shorter DFI and OS compared to the negative group in this population only (Figures 12.7 and 12.8). This reduction was due to a shorter time to distant metastasis as noted by the Kaplan-Meier survival curve (Figures Appendix 8, 9 and 10). No prognostic information was gained from detecting c-myc AAbs in the sera of screen-detected patients (Figure 12.3). The unequal numbers in the positive and negative groups may have contributed to similar tumour pathologies between the two groups despite differences in survival (Table 12.8).

Despite a significant correlation between the presence of c-myc AAb in the sera of our symptomatic breast cancer patients and tumour prognosis, when pooled into a panel, no association with prognosis was shown (Table 12.9, Figure 12.9). Earlier data from Cheung (Cheung 2001) contradicted our current result as c-myc AAb detection did not signify prognosis in his thesis.

c-myc oncoprotein was noted in the cytosol of breast cancer cells (Liao 2000, Review). It is either over-expressed or its oncogene amplified. The latter is a late event in breast carcinogenesis (Selim 2002) and could therefore account for poorer prognosis. IHC and PCR



have shown that the c-myc oncoprotein was over expressed in 45% of breast tumours whilst amplification of the c-myc oncogene was noted in 25% of tumours (Naidu et al, 2002). Furthermore both overexpression and amplification appeared to have a significant correlation with poorly differentiated tumours with poorer prognosis. Interestingly the authors failed to detect any relationship with either ER or LN status i.e. findings which were similar to ours. C-myc oncoprotein was also more readily seen in benign cases undergoing malignant change (Hehir et al, 1993). This again implicated c-myc in the pathogenesis and progression of breast cancer.

Table 3.3 highlights the various papers supporting (and some refuting) c-myc overexpression or oncogene amplification conferring a poorer prognosis in breast cancer patients. This was backed by the meta-analysis from Deming which confirmed its negative impact on prognosis in breast cancer patients (Deming et al, 2000). Our results therefore seem to confirm the growing body of evidence that c-myc confers a poorer prognosis in this group of cancer patients. As no multivariate analysis was performed we are unable to conclude whether c-myc AAb is an independent factor in determining prognosis. However, with such small size in each population, it is also possible that the result was spurious (type II error) or had arisen due to a play of chance due to multiple testing (type I error).

Our results in terms of prognosis were different from data presented by Cheung from the same unit as detailed in above section. Alterations made to the assay as noted in earlier chapter (Section 6.2.2) might result in these discrepancies. Current results may be more robust due to the alterations made in-order to reduce non-specific binding. The current data on prognosis for individual markers and panel correlate well with some units as noted in the published literature whilst contradicting others (Tables 3.1, 3.2 and 3.3). These differences

may be accounted by the multiple aspects of the studies involved as detailed in earlier paragraphs. Except for the c-myc AAb marker, the other markers showed no association with prognosis. Despite this limited prognostic information, the findings raise the future possibility that with further development, assays for AAbs could potentially be used as an adjunct to traditional methods of detecting primary breast cancers.

## Chapter 13

### Tissue Antigen and Circulating Antibody Correlation

### 13.1. CORRELATING TISSUE ANTIGENS AND SERUM AUTOANTIBODIES

As part of the research, tissue samples from women with PBC were analysed for the three antigens in our study. This work intended to correlate expression of tumour antigens in tissue and the corresponding AAb in serum. A comparison with previous work and other units was also detailed, further validating the data. The laboratory work for the IHC was performed by Dr Sarah Pinder and her team at the pathology department of Nottingham City Hospital, to whom I am grateful. The following is an outline of the procedure as performed by Dr Sarah Pinders team.

A standard Streptavidin biotin complex (AB) technique was performed. Breast cancer tissue was initially fixed in formalin and then embedded on paraffin wax. The sections were placed in 60°C incubator for 10 minutes and then de-waxed by immersing in 2 sequential xylene baths for 5 minutes each. It was re-hydrated by immersing in a series of 3 alcohol baths.

Endogenous peroxide activity was blocked by immersing the slides in 0.3% H<sub>2</sub>O<sub>2</sub> solution in methanol for 10 minutes then washed with tap water and rinsed with TBS. The samples were further blocked by applying 100µl of NSS (Diluted 1/5 in TBS) to each slide and incubating for 10 minutes to inhibit non-specific binding.

100µl of primary antibody was applied to each slide and incubated for 45 minutes. The primary antibodies were optimally diluted in NSS/TBS (MUC1: 1/300, p53: 1/50, c-myc: 1/125).

The following monoclonal antibodies were used as the primary antibody:

- MUC1 – Ma695 (IgG1 – Novacastra Laboratories Ltd)
- p53 – DO-7 (IgG2b - Novacastra Laboratories Ltd)
- c-myc – 9E10 (IgG1 – DAKO)

After washing in TBS 100µl of biotinylated secondary antibody (diluted 1/100 in NSS/TBS) was applied for 30 minutes at room temperature. The optimally diluted StreptABComplex was added to the section for 55 minutes and then washed in TBS.

In the final stages of the process, 100µl of DAB solution was added and the section washed before applying 100µl of copper sulphate solution. Haematoxylin was applied to the sections for 2-3 minutes, further washed, rinsed and dehydrated in alcohol before being mounted for reading.

Immunohistochemical staining was assessed by the author after training by the department. The staining was assessed on proportion of cells stained (scored 1 to 5) and the intensity of the staining (scored 0 to 3) (Harvey et al, 1999). Combined scores of more than 2 were taken as positive.

## 13.2. RESULTS

Thirty-four random PBC cases were chosen. IHC was performed to assess the presence of MUC1 and p53 antigens in respective tissue. For c-myc 10 samples were not analysed due to technical error and therefore only 24 tumour samples were available for assessment. Tissues were obtained during routine surgery for cancer.

Table 13.1. Correlation of immunohistology and ELISA for antigens and antibodies

	<b>MUC1 (n=34)</b>	<b>p53 (n=34)</b>	<b>c-myc (n=24)</b>
Positive IHC (Antigen)	17	4	10
Negative IHC (Antigen)	17	30	14
Positive ELISA (AAb)	9	10	7
Negative ELISA (AAb)	25	24	17
Positive both	7	1	5
Negative Both	14	21	12
Correlation	21 (62%)	22 (65%)	17 (71%)

Table 13.2. Correlation between Tissue Staining and Circulating AAbs to MUC1

<b>MUC1 staining</b>	<b>MUC1 AAb +ve</b>	<b>MUC1 AAb -ve</b>	<b>Total</b>
Negative	2	14	16
Positive	7	11	18
Total (N)	9	25	34

P = 0.1251 (non-significant)

Table 13.3. Correlation between Tissue Staining and Circulating AAbs to p53

<b>p53 staining</b>	<b>p53 AAb +ve</b>	<b>p53 AAb -ve</b>	<b>Total</b>
Negative	9	21	30
Positive	1	3	4
Total (N)	10	24	34

P = 1.0000 (non-significant)

Table 13.4. Correlation between Tissue Staining and Circulating AAbs to c-myc

<b>c-myc staining</b>	<b>c-myc AAb +ve</b>	<b>c-myc AAb -ve</b>	<b>Total</b>
Negative	2	12	14
Positive	5	5	10
Total (N)	7	17	24

P = 0.0850 (non-significant)

### 13.3. DISCUSSION

AAbs as detected by ELISA in the serum of 34 patients with known breast cancer were correlated with detection of corresponding antigens in the tissues of the same patients (Table 13.1). Our results indicated a 62% to 71% correlation between detecting the AAb using our ELISA technique and detection of the antigens in the corresponding patients breast cancer tissue with IHC. However analysis of the data using Fishers exact test showed no statistical correlation between the individual markers in tissue and the circulating AAb (Tables 13.2, 13.3 and 13.4).

Previous data from the unit (Cheung 2001), also failed to show any statistical correlation between MUC1 and p53 staining in tissue and the corresponding circulating antibody. There were no data on c-myc.

Published reports from other units have noted a correlation between p53 AAbs in the serum and its presence in the tissue (Mudenda et al, 1994). Review from Soussi noted although mutated p53 antigens were noted in 30% of breast cancer only half these expressed the AAb (Soussi 2000). Data for MUC1 shows only a partial correlation (Croce et al, 2003). There appears to be no data except ours as yet on c-myc AAbs in the serum and its corresponding antigen in the breast cancer tissue. Our overall results therefore add to the published data.



## Chapter 14

### All Groups

#### 14.1. ALL GROUPS

898 samples were analysed for the presence of AAbs to MUC1, p53 and c-myc in the serum. These samples were subdivided into their groups of origin i.e. Control (Normal and Benign), at-risk (fhx and ADH) and PBC cases (DCIS, screen detected and symptomatic).

Table 14.1. Frequency of AAbs at Mean + 3SD cut-of in each of the populations

	<b>MUC1 AAb</b>	<b>p53 AAb</b>	<b>c-myc AAb</b>	<b>Panel of markers</b>	<b>Any 2 AAbs</b>	<b>All 3 AAbs</b>
Control	3 (2.7%)	3 (2.7%)	5 (4.5%)	10 (9.1%)	1 (0.9%)	0 (0%)
(Normal)	3 (6.8%)	3 (6.8%)	4 (9.1%)	9 (20%)	1 (2.2%)	0 (0%)
(Benign)	0 (0%)	0 (0%)	1 (1.5%)	1 (1.5%)	0 (0%)	0 (0%)
At-risk	36 (9.4%)	21 (5.5%)	19 (4.9%)	70 (18.4%)	6 (1.5%)	0 (0%)
(Fhx)	31 (8.7%)	21 (5.9%)	19 (5.3%)	64 (18%)	6 (1.6%)	0 (0%)
(ADH)	5 (20%)	0 (0%)	0 (0%)	5 (20%)	0 (0%)	0 (0%)
All PBC	42 (10.3%)	85 (20.9%)	40 (9.8%)	143 (35.1%)	16 (3.9%)	2 (0.4%)
DCIS	6 (9%)	20 (30.3%)	4 (6%)	29 (43.9%)	2 (3%)	1 (1.5%)
Screen Detected	10 (8.4%)	37 (31.1%)	10 (8.4%)	51 (42.8%)	6 (5%)	0 (0%)
Symptomatic	26 (11.7%)	28 (12.6%)	26 (11 %)	66 (29%)	13 (5.8%)	1 (0.4%)

Table 14.2. Summary of frequency with 95% exact confidence interval and specificity of AAbs to tumour associated antigens at Mean + 3SD cut-off

	<b>MUC1 AAb</b> <b>N</b> <b>% (95% CI)</b>	<b>p53 AAb</b> <b>N</b> <b>% (95% CI)</b>	<b>c-myc AAb</b> <b>N</b> <b>% (95% CI)</b>	<b>Panel of markers</b> <b>N% (95% CI)</b>
Control	3 2.7% (0–6)	3 2.7% (0–6)	5 4.5% (1-8)	10 9.1% (4-15)
At-risk	36 9.4% (7-13)	21 5.5% (3-8)	19 4.9% (3-7)	70 18.4% (14-22)
PBC	42 10.3% (7-13)	85 20.9% (17-25)	40 9.8% (7-13)	143 35.1% (30-40)
P	P<0.0001	0.0006	0.0469	
Specificity	92.4%	95.2%	95%	83.6%

P (Kruskal-Wallis non-parametric test for significance) <0.05 signify the medians of the three groups (Control, At-risk and PBC) vary significantly

Fig.14.1. Distribution of all groups for the presence of MUC1 AAb

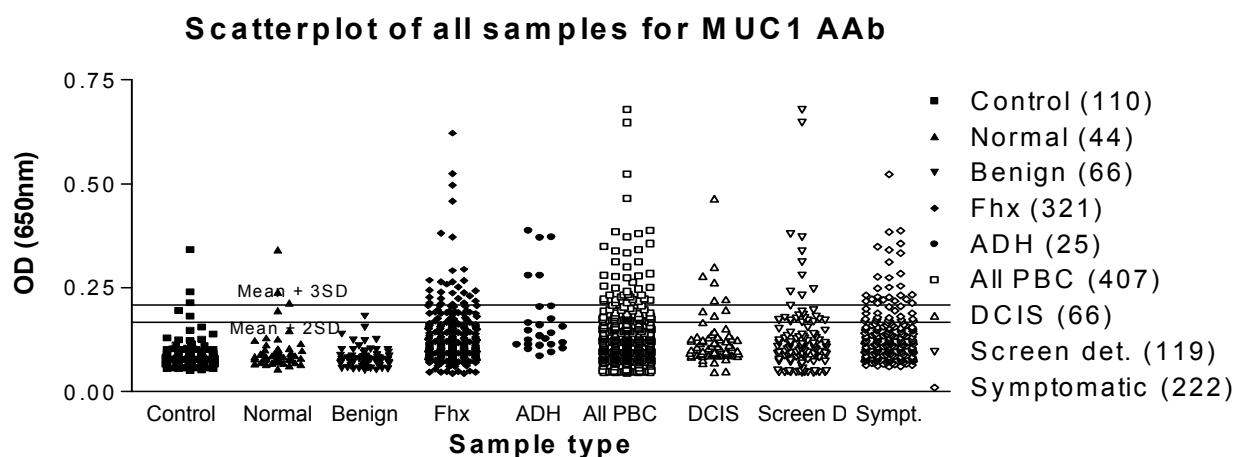


Fig.14.2. Distribution of all groups for the presence of p53 AAb

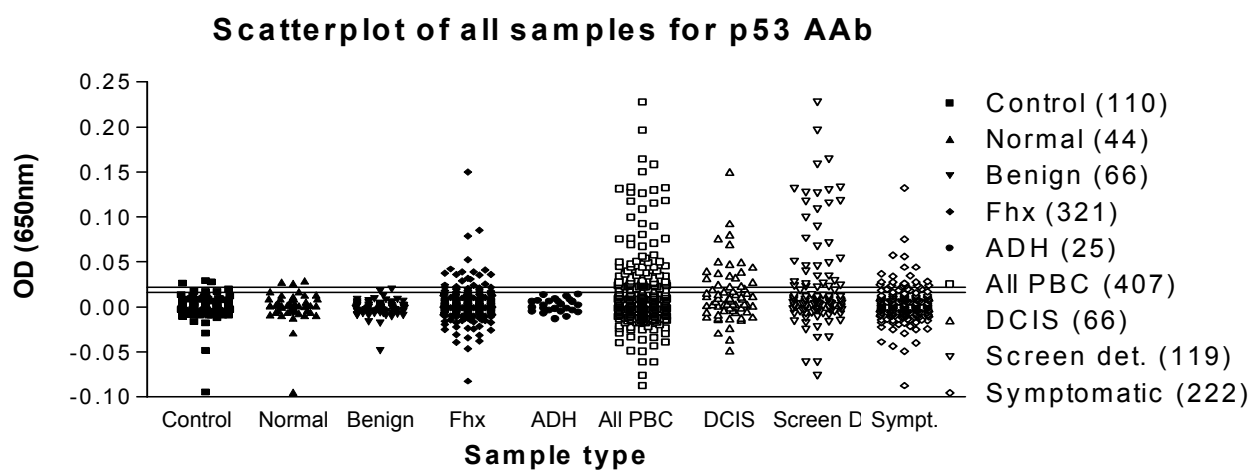
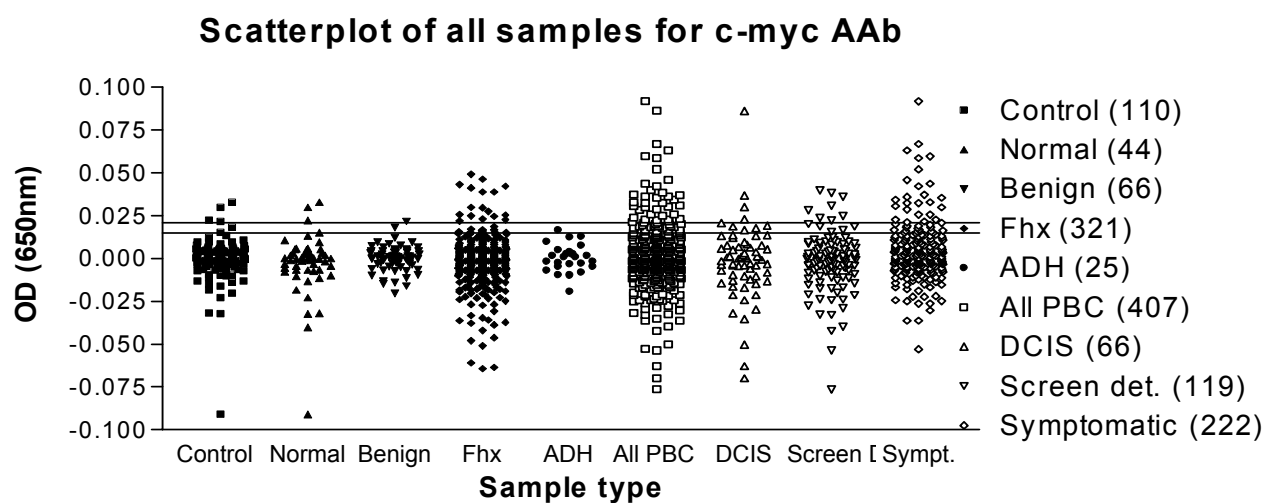


Fig.14.3. Distribution of all groups for the presence of c-myc AAb



## 14.2. DISCUSSION

AAbs to MUC1 p53 and c-myc were measured in 898 samples of various populations including Control samples that included sera from Normal and Benign groups, At-risk (fhx and ADH) and PBC groups using a prototype assay with 3 AAbs.

When reviewing the data for all the PBC samples we note that for both MUC1 and c-myc the prevalence of these markers were not statistically different between the different PBC cases i.e. DCIS, screen detected and symptomatic (Table 14.1). This may imply that humoral response to these mutated or over expressed proteins occur throughout pathogenesis of tumour cells. However p53 AAb production was greatly increased in DCIS and screen-detected cases, both known to have better prognosis, compared to symptomatic tumours. This implies that p53 mutation or overexpression resulting in a humoral immune response is an early event in breast carcinogenesis. It also supports our hypothesis that a detectable immune response is an early event in cancer evolution. The ability to detect this marker may be useful in a panel with technically developed assays as a method for screening women for carcinogenesis.

Our results attempted to ascertain the prevalence of each of the 3 tumour markers in at-risk and primary breast cancer (pre and invasive cancers). To increase the sensitivity of attaining a positive result we also determined the prevalence to a panel of all 3 markers. This showed that 35.1% of all primary breast cancers were positive for at least one of the 3 markers at the higher cut-off value (Tables 14.1 and 14.2). However this method also increased the possibility of reducing the specificity of the tests (Table 9.13 and 9.14), as inevitably false

positives will be more likely; the specificity of MUC1, p53 and c-myc AAbs were above 90% each but as a panel it was 83.6% (Mean + 3SD).

Our panel result for at-risk cases was 18.4% (Tables 14.1 and 14.2), which was twice that for the Control population (Tables 14.1 and 14.2). The three assays were all prototypes, which despite the alterations made to previous assay still had significant background signal due to non-specific binding. This was evident from the signal elicited from the negative control VOL. On many occasions the signal from the negative control was greater than the actual antigen.

If the relatively high signal, a significant proportion of which we believe to be due to non-specific binding can be eliminated then the small differences seen between the control and at risk group might in future provide a real signal for a true 'at risk' population. Clearly further development and research is required to prove this.

Of the 13 at-risk cases that have progressed to breast cancer only two had a positive result for an AAb prior to the diagnosis of breast cancer. The low sensitivity of the assay to detect occult tumour inhibits its use in screening. This is further supported by the high false positives in the at-risk group.

If more than 1 or even 2 positive marker is used as a criterion for establishing a positive result the specificity increased to clinically acceptable levels (Tables 9.13 and 9.14). This however inevitably reduced the sensitivity (Tables 9.11 and 9.12). As the sensitivity of the assay in screening was already low, reducing this further would undermine any value of the assay.

As mentioned before, screening tools require a high specificity to reduce false positives that would overburden the system during screening. False positive results would also cause distress to patients who undergo unnecessary investigations. But since current screening for breast cancer is limited by factors described in earlier chapters, AAb marker detection either singly or in a panel may be useful as an adjunct in those cases where screening is difficult. This group includes the very young women, at-risk men and women with implants and on HRT. For diagnostic purposes since no one marker appears to be sensitive enough a panel of markers may be necessary.

Other authors have also commented on this need to using a panel of AAb markers to enhance the detection rate. Zhang and colleagues have reported that individual markers only gave a frequency of 15 -20% but within a panel, this frequency raised to 44% - 68% (Zhang et al, 2003). They further noted that different cancer types have different profiles of marker rise thus aiding diagnosis. In a more recent paper, addition of p53, c-myc and p16 AAbs to a panel gave a positive result in 44% (Looi et al, 2006). These results provide strong confirmation that our own panel detection of 35.1% was a true biological finding. However our data had shown limited value in prognosis although the c-myc AAb marker may be promising.

Overall our data had supported our initial hypothesis that an immune response is an early event in cancer formation and detection of this response can lead to early cancer detection. We have managed to establish an in-house ELISA technique for the detection of AAbs to MUC1, p53 and c-myc. The method was moderately reproducible and results complimented other published data enhancing its reliability, although marked differences from previous data from the unit were also noted. These differences may be due to the enhancement of the



current assays. Detection of the tumour markers in screening, diagnosis and prognosis had been shown to be very promising but more follow up of current cases is required. Further technical development of the assays will also be necessary before they could be useful in a clinical setting and this work is currently ongoing.

## Chapter 15

### Summary, Criticism and Conclusion

## 15.1. SUMMARY

Breast cancer is formed by multiple genetic changes that result in qualitative and quantitative alterations in individual gene expression (Watson, 1996). These alterations result in the formation of tumour associated antigens by mutation, over expression by either gene amplification or gene over expression and altered expression (Storr et al, 2007.). Our hypothesis is that an immune response to this altered antigen is an early event in tumour formation. The detection of the antibodies, which are the amplified signal to these antigens, can therefore help in the early detection of breast cancer. The current restrictions in screening for breast cancer indicate the need for alternative or adjunctive methods.

Using tumour markers as a screening, diagnostic and prognostic tool is not new but current methods use antigens of tumours, which are limited by their poor sensitivity and specificity due to low tumour burden in the early critical stage of tumour progression. However the concept of using AAbs of tumour antigens allows us to amplify signal and thereby enable detection of these tumours at an earlier stage. Our study intended to focus on the screening, diagnosis and prognosis aspect of breast tumour markers. Early detection even of recurrent disease may have an impact in both early treatment and survival (Jager et al, 1994; Nicolini et al, 1997, Lu et al, 2008) although this is widely refuted by other studies (Khatcheressian et al, 2008; Jatoi et al, 2005).

Both MUC1 and p53 AAbs are the most commonly studied markers in breast cancer (Tables 3.1 and 3.2). The first AAb to tumour-associated antigen to be detected was to p53 in 1982 (Crawford et al 1982). Since then the concept of AAbs as markers of breast cancer has led to multiple AAb detection, each with varying presence in the serum of patients with PBC.

Although none of the markers have yielded a clinical role, their potential to do so remains a possibility. The various AAb markers in serum of breast cancer patients in the current literature include Mammaglobin (Watson et al, 1996), survivin (Goksel et al, 2007; Yagihashi et al, 2005, Megliorino et al, 2005), livin (Yagihashi et al, 2005), NY-ESO-1 (Bandic et al, 2006), Annexin XI-A (Fernández-Madrid et al, 2006), Endostatin (Balasubramanian et al, 2007), Hsp90 (Pick et al, 2007), p62 (Rolland et al, 2007) and koc (Zhang et al, 2003).

MUC1 (Albrecht et al, 2007; Mommers et al, 1999), p53 (Moll et al, 2001), Annexin XI-A (Fernández-Madrid et al, 2006) and Endostatin (Iughetti et al, 2001) antigens are examples of mutations of the original antigens, whilst c-myc (Deming et al, 2000 Review), HER2 (Di Fiore et al, 1987), p62 (Rolland et al, 2007), mammaglobin (Watson et al, 1996) and koc (Müeller-Pillasch et al, 1997) are over-expressed. c-myc overexpression is due to either gene amplification (Blancato et al, 2004) or over expression of protein (Selim et al, 2002). Survivin (Goksel et al, 2007; Yagihashi et al, 2005, Megliorino et al, 2005), an anti-apoptic protein, livin (Yagihashi et al, 2005), NY-ESO-1 (Bandic et al, 2006) and Hsp90 (Drysdale et al, 2006) are ubiquitously expressed in cancer cells.

Some of the above expressed markers give prognostic information. Mammaglobin is a glycoprotein that is selectively expressed by breast cancers (Watson 1996) although minimal amounts are noted in normal breast tissue. Current data has shown its presence in metastatic LN but absence in disease-free glands (Watson, 1999). This discrimination may enable its use in prognosis. Furthermore mammaglobins presence in the serum of breast cancer patients may act as a surrogate marker of disseminated cancer cells (Bitisik, 2005).

AAbs to Hsp90 can be detected in the sera of breast cancer patients where they can predict poor prognosis (Pick et al, 2007). The presence of Hsp90 AAbs was an independent prognostic factor in multivariate analysis. Contrary to the above, detection of AAbs to endostatin has been shown to favour improved prognosis (Bachelot et al, 2006) possibly due to a natural immune response to endostatin.

Our study was a continuation of previous data from the unit, which had used MUC1, p53, HER2 and c-myc antigens to detect the corresponding AAbs using ELISA (Cheung, 2001). We therefore persisted with the original markers to assess their role in screening, diagnosis and prognosis. A breadth of literature is available for both MUC1 and p53 as either antigens or their corresponding AAbs (Tables 3.1 and 3.2). p53 antigens are the result of gene mutation whilst mutated MUC1 arises from post translational changes. They therefore occur at different phases of cell cycle. Data on c-myc oncoprotein or AAb are sparser compared to the two above. However its value as a marker is noted in its detection in higher grade breast tumour (Deming et al, 2000). It is also over-expressed in tumour cells and therefore differs from p53 and MUC1. All three markers have been shown to prognosticate breast cancer, although this is debateable for p53 and MUC1 (Tables 3.1 and 3.2). For c-myc the majority of evidence indicates that it is associated with poorer prognosis breast cancer (Table 3.3). The three antigens therefore compliment each other within a panel of markers in terms of diagnosis and prognosis.

The detection of markers either as antigens or as AAbs within a panel greatly enhances the sensitivity of the markers to detect breast and other cancers as detailed in two reviews by Zhang (Zhang, 2004 and 2007). Different cancers may require different combination panels. Current literature testifies to the multiple combinations of markers used in panels. Common

marker combinations for breast cancer include p53 and c-myc with addition of MUC1 and HER2 (Cheung, 2001), HER2, NY-ESO1, BRCA1, BRCA2, and MUC1 (Chapman et al, 2007) cyclin B1, survivin, p62, koc and IMP1 (Zhang et al, 2003) and survivin (Megliorino et al, 2005). Data from Megliorino showed that survivin was noted in only 8.4% in all cancers with breast, lung, lymphoma and hepatocellular cancers showing higher prevalence compared to normal human serum. Combining p53 and c-myc to survivin within a panel increased the detection rate to over 26.6% in breast cancer (Megliorino et al, 2005). This may have clinical potential.

Novel combinations include koc and p62 (Zhang et al, 2001) and survivin and livin (Yagihashi et al, 2005). Sensitivities to breast cancer for the panels range from 16% (Koc and p62) (Zhang et al 2001) to 82% (MUC1, p53, c-myc and HER2) (Cheung, 2001). The difference in sensitivities is dependent on individual markers within the panel and cut-off value used.

Sensitivity and specificity data in our study had shown individual marker sensitivity ranging from 9.8% for the c-myc AAb to 20.9% for p53 AAb in PBC samples. The panel sensitivity was 35.1%. The specificity however was over 90% for individual markers, but reduced significantly to 83% for the panel. Adding another marker, as detailed in earlier paragraph, may increase the sensitivity of the panel still further. To increase the specificity the cut-off value can be increased to Mean + 4SD or be more dependent on the specificity required (Table Appendix 3).

Data comparing PBC with At-risk populations (Figures Appendix 1, 2 and 3) for individual markers had shown increased markers in PBC samples for p53 and c-myc AAbs. The MUC1

AAb sensitivity between the two groups was not different and therefore its potential use in a panel to differentiate between those with breast cancer and those without is limited. Removing the MUC1 AAb marker from the panel would increase the specificity to 93.6% without altering the sensitivity of this panel unduly (29% from 35.1% for PBC) (Table Appendix 3).

Other combinations of the panel are also highlighted in Table Appendix 3. This demonstrates various sensitivities and specificities of the panel for PBC samples, thus fine tuning the panel to meet specific clinical needs. Increasing the cut-off value to Mean + 4SD (Table Appendix 3) would greatly increase the specificity to a clinically viable level above 97%. Although the panel sensitivity was only 20%, this was due to only MUC1 and p53 AAbs. No c-myc AAb was detected in PBC panel at this level. Therefore substituting c-myc for another marker may potentially greatly increase the overall sensitivity and specificity. This is very promising for further studies.

The ELISA method of AAb detection as used in our study was a prototype assay with only 3 AAbs used within the panel. It was redeveloped and optimised from previous ELISA techniques used in our department. We had managed to produce an antigen in a vector that allowed its biotinylation. This biotinylation enabled the protein to be immobilised onto the neutravidin-coated microtiter plates and therefore may become more accessible to the AAbs during the ELISA assay (Cordiano et al, 1995). The avidin-biotin interaction has one of the highest association constant yet reported ( $K_a = 10^{15} \text{M}^{-1}$ ) (Green, 1975). Such strong interaction allows the avidin-biotin complex to remain intact despite multiple washings (Cordiano et al, 1995). In our assay, we substituted avidin for neutravidin due to lower non-specific binding of the latter (Hiller 1987).

We attempted to ascertain the reliability of the new technique by assessing its reproducibility and comparing signal difference between controls and cancer patients. Reproducibility was assessed using a well-validated method i.e. the Bland Altman methodology to calculate the CR. Our results indicated a reliable degree of reproducibility but the level of reliability was dependent on the population analysed and the antigen used i.e. the MUC1 AAb appeared more reproducible than the p53 AAb and benign population more reproducible compared to the other groups analysed. We are not aware of the exact mechanisms that may account for this although it is postulated that current assay techniques elicit a high background signal using the vector only lysates, hence the MUC1 AAb marker was more reproducible compared to either p53 or c-myc AAbs; assays of both requiring VOL as a control. This background signal can overwhelm and mask the positive signal, which may be much lower than the background signal. Therefore minor alterations during repeat assays can lead to misleading results.

Repeat thawing and freezing may also potentially damage individual samples resulting in poor reproducibility. It is therefore not possible to conclude whether it was the technique itself or at least in some part the samples contributed too. Obviously either of the conclusions can reduce the reliability of the attained results and therefore it is imperative to both further enhance the technique and specifically test the effects of multiple freeze thaw cycles.

Multiple washings with buffer solution were intended to reduce the background signal. The number of washings may need to be increased to further reduce the background signal although repeated washings may also alter antigen attachment to the ELISA. This could therefore result in inaccurate results. Either of the two hypotheses would however need to be



tested. Our results indicate that although it is difficult to be absolutely certain of individual results due to the moderate reproducibility and hence reliability of the assay, the general trend in each population appeared to correlate well with published data from other units around the world. This gives confidence to the overall results and technique used in this study. As mentioned before much further work is required to fine-tune the assay to improve individual results. This will become obvious with more reproducible assays.

Comparing signals between Controls and PBC samples noted significantly increased signal for all 3 markers in the PBC group compared to the Control at the lower Mean + 2SD cut-off value. At the higher cut-off value, increased signal for the c-myc AAb did not reach statistical significance. This may have been due to low numbers in each population. Furthermore the symptomatic group; a subgroup of PBC, did actually show increased c-myc AAb frequency compared to Control (Figure 11.4). It may suggest that c-myc AAb is not prevalent in earlier disease. The overall data therefore supports the assays reliability in detecting increased AAbs in cancer cases compared to the Control.

Our data on serum AAbs and its corresponding antigens in the tissue as detected using IHC showed some correlation between the two techniques for all 3 AAbs although not statistically significant. However this cannot be assumed to validate the presence of AAbs for our ELISA assay as only p53 has shown good correlation in the published literature (Mudenda et al, 1994). MUC1 only shows a moderate correlation (Croce et al, 2003) whilst data for c-myc is still not available.

Having 2 cut-off values i.e. greater than Mean + 3SD and also the Mean + 2SD value enabled us to compare the usefulness of the test at various sensitivities and specificities. In detecting

occult tumours our results showed that although the specificity of the ELISA was above 80%, the sensitivity was very low and therefore the current assay is not clinically useful in screening (Table 9.12). Setting the positive cut-off value as 2 or more positive AAbs would further increase the specificity; an imperative in screening, but will further reduce the sensitivity (Tables 9.12 and 9.14). This lack of sensitivity in determining breast cancer in the at-risk family history group was noted by the detection of only 2 tumour markers (in 2 individuals) in 13 women who had progressed to develop breast cancer prior to the diagnosis of breast cancer at the higher cut-off value (Table 9.10). Although the cause of this lack of sensitivity of our markers is not known, the mean time to diagnosis from sample donated of 18 months may have been too long for accurate assessment although detection of positive markers was up to 7 years in one individual and mean time to diagnosis was 43.5 months for the 2 positive cases. Detecting tumours 7 years before diagnosis makes the assay clinically unhelpful, as it would be difficult to locate the lesion with current means although the expansion of MRI use may resolve some of these issues. The result suggests a spurious result although a true positive cannot at this stage be ruled out.

Comparing current with previous data from the unit (Cheung, 2001) was difficult as only two patients were noted to have developed breast cancer during the initial study period. However one of the two was noted to have sero-converted to the p53 AAb six months prior to cancer diagnosis. Both individuals in our study who had positive markers prior to diagnosis of cancer progressed to DCIS, which is a pre-invasive tumour and microscopically the cancer cells are contained within the basal lamina. Explanation for this finding may be that both MUC1 and p53 AAb sero-conversions are the first event in carcinogenesis. It is also a possibility that the result is spurious due to low numbers analysed in this cohort of women. Future work must concentrate on increasing the sensitivity of the ELISA technique if

screening is an indication. Furthermore, samples at shorter interval (i.e. reduced mean time to diagnosis) appear to be indicated in future study.

Our results on women with benign breast disease as a single group showed very few with any raised tumour marker despite reports of the presence of oncoproteins in the tissue (Sirotkovic-Skerlev et al, 2005; Croce et al, 1997; O'Connell et al, 1998; Keohavong et al, 2004). Results for Benign cases differed markedly from the original study. p53 AAb detection was significantly higher in the original study compared to the current thesis. It may be that the current assay is more reliable with reduced false positive due to reduced background signal. This was the basis of the alterations made to the assay for the current thesis.

Our results for the at-risk individuals showed MUC1 AAb sensitivity higher than p53 or c-myc AAbs at both cut-off levels (19.6%, 11% and 8.7% respectively for Mean + 2SD and 9.4%, 5.5% and 4.9% respectively at the Mean + 3SD). This contrasted with earlier data from Cheung (Cheung 2001) where p53 AAb sensitivity was the highest. MUC1 is secreted by normal healthy individuals but in low amounts (Bjerner et al, 2002). This may account for some of the MUC1AAbs noted in the at-risk group.

The at-risk group had significantly raised MUC1 and p53 AAbs compared to the control (Figures 9.1 and 9.2). c-myc AAb detection in the at-risk population did not reach significance when compared with the Control group. This suggests a population, which is beginning to sero-convert to the antigens in our study. The at-risk group may harbour occult tumour, which is not yet diagnosed, and therefore this sero-conversion is the first indication of potential malignancy. This supports our initial hypothesis that an immune response is an

early event in cancer evolution. Detection of this immune response via serum AAb detection is the basis of the thesis. Further support of this is that AAbs to p53 and c-myc in the PBC group were significantly higher than at-risk (Figures Appendix 1, 2 and 3). This implies a stepwise increased immune response through groups that are increasing in malignant potential. The difference in the markers that are raised highlights inherent flaws in the assay which makes individual results difficult to interpret although establishes trends very well.

We demonstrated PBC cases could be sub-divided into pre-invasive and invasive cancer. The two are biologically different with considerable difference in treatment and prognosis. The latter group can also be further divided in screening and symptomatic cancer. Although these are biologically similar tumours (Cowan et al, 1997) treatment and prognosis differs as noted in Table 11.4 and Figure 11.10.

The presence of AAbs in the sera of pre-invasive tumours (i.e. DCIS) suggests these antigens are detected by the immune system. Shimokawara had detected an excess of infiltrating B lymphocyte in the stroma of DCIS (Shimokawara et al, 1982). It is believed this plays a possible role in AAb production in this pre-invasive disease. Presence of AAb in such early phase of breast carcinogenesis supports our hypothesis that an immune response to tumour-associated antigens is present at an early period of cancer evolution. Therefore if these AAbs are detected they can lead to the detection of early occult disease. AAbs are more readily detected than the corresponding antigen due to their amplified state.

Results of our DCIS tumours showed positive markers for all three AAbs, with p53 AAb the most prevalent at 30.3% (Section 11.2). MUC1 and c-myc AAb sensitivities were similar while the panel of all 3 markers gave a prevalence of 43.9%. Both p53 and MUC1 AAb

sensitivities were significantly higher compared to Control (Figure 11.4 and 11.5). However c-myc AAb sensitivity was similar to control. This may be accounted for by c-myc over-expression noted in larger, more aggressive tumours, quite unlike DCIS (Deming et al, 2000).

AAb detection in DCIS differed significantly from previous data at our unit from Cheung (Cheung, 2001) who had detected minimal AAbs in DCIS cases. However, more recent results from Chapman (Chapman et al, 2007) compares favourably with our own. Chapman also measured AAbs to a panel of bacterially produced antigens, which also included p53 and c-myc. She had however used a different method for coating the antigen to the ELISA plate. Despite this difference in ELISA assay the results for p53 and c-myc AAbs were similar. This similarity between the later two studies from the unit may indicate more reliable data in our study compared to those of Cheung.

IHC of DCIS tissue samples by O'Malley (O'Malley 1994) had shown p53 mutated antigens were present in 33% of comedo-type DCIS. O'Malley failed to detect any mutated p53 in non-comedo type. This significance is noted, as the comedo form of DCIS is more aggressive than the non-comedo form. The presence of p53 antigens may therefore indicate high risk of invasion in these pre-invasive cancers. Our own data failed to highlight any association between serum AAb and grade of DCIS (Table 12.1).

Cancers noted via the NHSBSP tend to be more in situ and if invasive, to be smaller, of lower grade and to have invaded vessels, peri-neural spaces and lymph nodes less frequently (Cortesi et al, 2006). This favourable tumour parameter is illustrated in Table 11.4.

Our data showed very similar detection rates for screen detected as for DCIS samples i.e. 8.4% for MUC1 AAb, 31.1% for p53 AAb and 8.4% for c-myc AAb (Table 14.1). For the panel of these AAbs the sensitivity was 42.8%. This similarity between DCIS and screen-detected would be expected as majority of DCIS is detected during routine mammographic screening. The presence of AAbs either single or within a panel did not correlate with tumour grade, stage or size or other tumour specifics (except for the p53 AAb where its presence noted a higher grade and vascular invasion). Furthermore as was the case with DCIS AAb detection either as a single positive result or within a panel did not predict survival or recurrence.

Symptomatic tumours were larger, had a higher grade, were more likely to invade vessels and were generally of poorer prognosis when compared to screen detected cases (Table 11.4). Furthermore the former was less likely to be ER positive compared to the latter, again another feature of poorer prognosis. This translated well into a shorter survival from diagnosis in the symptomatic group as noted by the survival curve (Figure 11.10). While the longer survival could simply be due to lead-time randomised controlled trials of mammographic screening have confirmed a significant reduction in breast cancer mortality and an increase in survival (Tabar et al 1989; Tabar et al 2001; Tabar et al 2003; WHO handbook, 2002; Nystrom et al, 2002).

MUC1 AAb prevalence in our assay for symptomatic patients was 11.7% (Table 11.5). This was similar to our previous data on screen detected, DCIS and even healthy at-risk patients (Table 14.1). Presence of this AAb did not predict recurrence, survival or tumour pathology.

p53 AAb prevalence in our assay for symptomatic patients was 12.6% (Table 11.5). This was significantly lower than compared to DCIS (Figure Appendix 7). There was no significant difference in tumour pathology (Table 12.7), DFI (Figure 12.6) or OS between tumours that elicited a p53 humoral immune response and those that were negative for p53 AAbs. The marked reduction in p53 AAb in invasive cancers compared to DCIS may indicate that an immune response to p53 is an early feature of breast cancer.

c-myc AAb prevalence in our assay for symptomatic patients was 11.7% (Table 11.5). Although there were no discernible difference in tumour pathology between those with c-myc AAbs and those without (Table 12.8), the c-myc AAb positive population had a shorter survival compared to the negative group (Figure 12.8). This reduction was due to a shorter time to distant metastasis as noted by the Kaplan-Meier curves (Figures Appendix 8, 9 and 10).

Presence of c-myc AAbs may therefore indicate poorer prognosis as noted from our data. This result confirmed meta-analysis suggesting c-myc amplification in breast cancer indicates a poorer prognosis (Deming et al, 2000). If confirmed, then detection of c-myc AAbs could have clinical implications. However it is also probable that our data is a spurious result due to multiple statistical tests being performed (Type I error) or due to low numbers in each population analysed (Type II error). It will require further investigation with larger number of samples and repeat assaying with sound reproducible data before any actual usage can be determined.

When data for all three AABs in symptomatic breast cancer was pooled together as a panel, a positive panel did not signify prognosis (Table 12.9, Figure 12.9). The overall prevalence of any one marker within the panel was 29.7% (Table 11.5).

Although individual marker detection in patients with established breast cancer yielded low prevalence they were higher in general than Control populations (Figures 8.7, 8.8 and 8.9). These results compared favourably with previous data from our unit (Cheung, 2001; Chapman et al, 2007) but the value of single AAb assays in diagnosis and screening appears of limited potential. This can be significantly improved by combining the AAb markers into a panel. This greatly enhanced the detection rate to 35.1% of all breast cancer (higher in specific groups).

Chapman (Chapman et al, 2007) had shown panel sensitivity for PBC cases greater than 50%. However the panel consisted of five AABs with the cut-off at the lower Mean + 2SD value. Panel sensitivity from Cheung (Cheung, 2001) was significantly higher with an overall sensitivity for all PBC cases of 74%. This result however again was at the lower cut-off value of Mean + 2 SD. The two assays were however different. These differences in assay methodology may account for the differences noted in the results.

Data on sequential samples showed that most samples remained sero-negative, although some patients in both fhx and PBC (ATAC) group showed alterations to their AAb status (Figures 10.1 to 10.6). This could imply a true event resulting from impact of treatment or progression of disease, or a natural variation within individuals over time. Alternatively it may signify inherent flaws of the assay in regards to reproducibility. With so few cases for fhx and PBC group undergoing sequential sampling, it is difficult to conclude from the available data.



Although our data do not support clinical use of individual markers, as a panel its value may be as an adjunct to other more traditional methods of diagnosis i.e. mammograms or newer methods such as PET or MRI. AAb detection may further be enhanced in selected groups of patients such as male, at-risk, young women or those with implants for whom mammograms may not be possible or reliable.

The crucial role of predicting breast cancer in at-risk women do not appear to be justified using the current assay format since we failed to detect any tumour marker rise in 11 of 13 patients who developed breast cancer in their pre diagnosed blood samples using the higher cut-off value. The reason for this failure must be addressed in future studies. On the other hand in the two we did detect AAbs, the lead-times were significant. Enhancing the ELISA method and / or measuring more frequent serial samples to attain shorter lead-time between sample donation and breast cancer detection and increasing the number of assays in the panel may all be warranted. This has obvious consequences with regards to resources needed and patient consent to more frequent blood donation. Furthermore the value of a very short lead-time to breast cancer diagnosis must also be justified with regards to OS.

## 15.2. CRITICISM

The current thesis was a continuation of previous work at the unit (Cheung 2001). Earlier data had shown greatly enhanced AAbs to a panel of 4 AAbs to the antigens; MUC1, p53, HER2 and c-myc) compared to current literature. However the assay used at the original period lacked specificity due to non-specific binding and therefore the ELISA assay was enhanced to reduce non-specific binding as detailed in chapter 6.

Due to shorter follow up period only two patients from the at-risk group developed breast cancer. Data from these two patients had failed to detect a meaningful rise in AAb prior to cancer development. This thesis aimed to use the enhanced assay with a longer follow up with more cases to establish the role of AAb markers in screening, diagnosis and prognosis of breast cancer.

However certain drawbacks were noted throughout the current study, highlighted in respective chapters. Main criticism of the data is the low number of patients used as Controls. Lack of Normal sample numbers (only 44) resulted in adding Benign cases to our Control population. We do not have any details of the histology in this group of Benign cases. This non-malignant Control group was therefore the combination of both Normal and Benign cases. Current literature has demonstrated both groups have small and insignificant amount of antigens and AAbs to the studied markers (Bjerner et al, 2002; Keohavong et al, 2004; Balogh et al, 2006; O'Connell et al, 1998). We were therefore able to combine the groups to increase the population size. The larger size established more accurate cut-off values. The combination of both groups was established in the previous work at the unit and further continued by the current author as part of the unit protocol.

Although the literature does not suggest the two populations are different in terms of detection of AAbs in the serum, biologically the two groups are different. The impact of this biological difference in combining the two is not ascertained in establishing cut-off values. Furthermore both populations consisted of samples of varying age. We attempted to establish whether age of sample affected signal (Chapter 7). Our data confirmed no significant signal difference between the older and recent Normal and Benign samples for any of the AAbs.

However data for older and recent PBC samples (Chapter 8) contradict the above with p53 AAbs showing reducing signal for the older samples. This may have been due to small number of cases in the subgroups (type II error) or to false positive result in multitude of data analysis (Type I error). A larger Normal sample population collected at the same period of study would minimise such errors as was done by Chapman in her more recent study from our unit (Chapman et al, 2007).

Our assay for both p53 and c-myc AAbs had a negative control (VOL) for each individual sample (Section 6.2.2.2.2). The VOL control enabled to correct any non antigen-specific binding results for the above AAb assays. However the MUC1 AAb assay did not have a negative control. It was therefore impossible to fully establish if any individual samples tested for positive for the MUC1 AAb were truly binding to the antigen and not due to non-specific binding to the plate.

Reproducibility as assessed by the Bland and Altman CR (Table 8.1) was only partial as the data suggested that some samples vary by greater than the positive cut-off values. Triplicates whose CV value was less than 10% confirmed intra-assay reproducibility. Greater variation than 10% deemed the result void and sample re-tested. However inter-assay variation was not monitored regularly. This may have been enhanced by repeating all positive results on four separate occasions as detailed in the study by Chapman (Chapman et al, 2007). The sample was deemed positive if 3 out of 4 repeat samples were positive.

Our original study design included 4 AAbs as previously studied by Cheung (Cheung, 2001). However at the start of the study period HER2 antigen was not available for analysis and therefore our panel measured only 3 AAbs. The low number of AAbs measured by the panel

reduced the sensitivity of the panel and may account for some of the discrepancies between the two studies. Increasing the number and type of markers in the panel would greatly increase the sensitivity of the panel. Recent published data has shown greater than 20% sensitivity to newer individual markers including survivin (24%) (Yagihashi et al, 2005), Annexin XI-A (19%) (Fernández-Madrid et al, 2006) Endostatin (66%) (Bachelot et al, 2006) and Hsp90 (37%) (Pick et al, 2007). For all these markers, the positive cut-off value was similar to our data and set at the higher Mean + 3SD. Combination of these markers within a panel would greatly enhance the sensitivity and possible clinical use of the panel. However the specificity of these markers as individuals and within a panel would significantly impact any clinical role.

### 15.3. CONCLUSION

We have developed a fairly reliable and reproducible assay for the detection of AAbs to the tumour associated antigens, MUC1, p53 and c-myc. This technique was used to ascertain the prevalence of each of the 3 AAbs as serum tumour markers in various populations i.e. at-risk and primary breast cancer; both pre and invasive cancers. The role of these tumour markers in screening, diagnosis and prognosis of breast cancer was elicited.

Our data supported our hypothesis that an immune response resulting in AAb production is an early event in cancer evolution. Our panel had detected higher levels of markers in early disease and those at risk compared to the Control population. The data on screening had shown fairly high specificity especially as cut-off values were increased but very low sensitivity. The assay is currently not yet ready to be used to screen the at-risk population. Its value therefore may be as an adjunct with other more traditional methods of screening,

especially in those for whom current traditional screening methods i.e. mammograms are limited at the present time.

For diagnostic purposes a panel of markers may be useful in the diagnosis of breast cancer in pre-invasive, screen-detected or symptomatic form of the disease as there was clearly a trend towards higher detection in those samples with known malignant disease as compared to non-malignant samples. The sensitivity of individual markers in cancer was not yet high for any clinical purpose but as a panel, more than a third of cancers had a raised marker. The specificity of the panel was however only 83%, which may be increased by removing the MUC1 AAb or increasing the cut-off value further. The development of the panel is promising as an adjunct to other means of clinical diagnosis. This is especially true as further improvements in reducing assay background signal are made.

The value of the markers as individuals or as a panel to aid prognosis is not yet established. Only the presence of c-myc AAb appeared to signify a poorer prognosis in terms of OS and recurrence. However within a panel, the prognostic value of c-myc AAb was overridden and therefore the panel of AAbs did not give any data on the prognosis of patients with breast cancer.

Future development will need to assess other AAbs within a panel of markers as well as alleviating some of the previously mentioned problems of the assay such as high background signal, which results in less than perfect reproducibility. Addition of other markers to the current prototype assay may yield higher individual and therefore panel marker detection and its use in screening may become an option. Furthermore ongoing research by other members of the research group is currently investigating differences in protein structure and

immunogenicity between batches of antigens, which might also affect the sensitivity and specificity of these assays. Also, as detailed before, the current markers already give some information on prognosis and future refinement of this assay will enhance this aspect of the panel usage.

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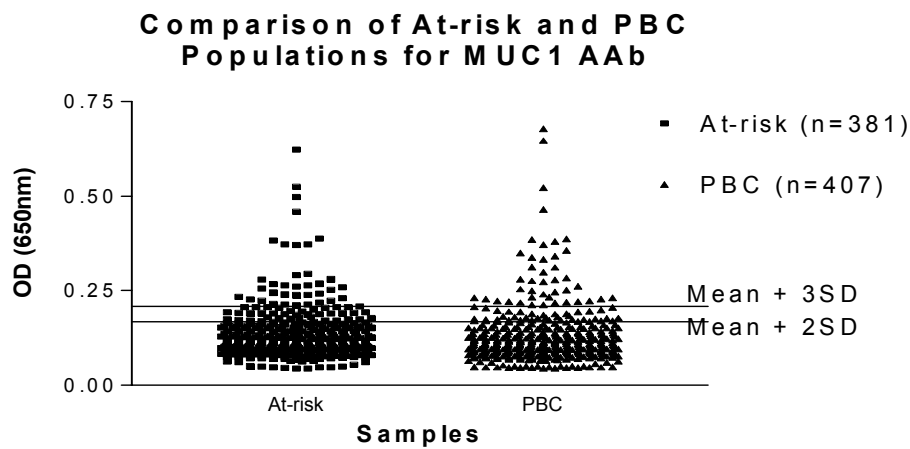
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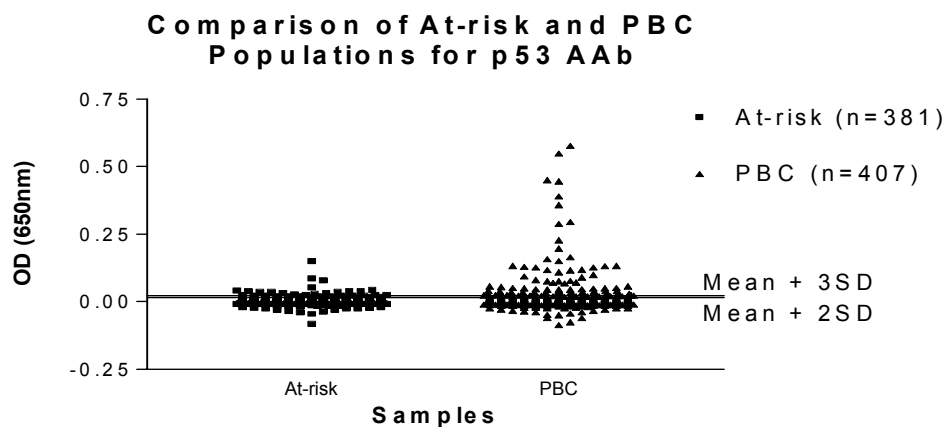
## APPENDIX

Fig. Appendix 1. Comparison of PBC and at-risk populations for MUC1 AAb



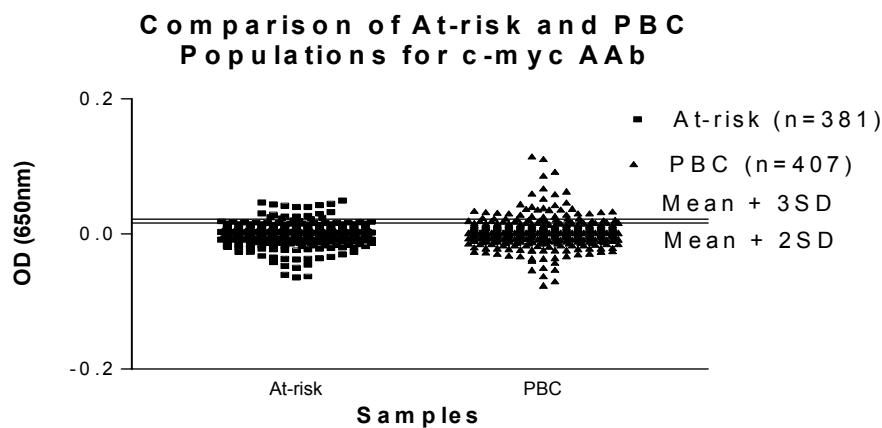
Median OD compared with non-parametric Mann-Whitney test for significance  $p = 0.1633$

Fig. Appendix 2. Comparison of PBC and at-risk populations for p53 AAb



Median OD compared with non-parametric Man Whitney test for significance  $p = 0.0089$

Fig. Appendix 3. Comparison of PBC and at-risk populations for c-myc AAb



Median OD compared with non-parametric Mann-Whitney test for significance  $p = 0.0167$

Fig.Appendix 4. Local recurrence in screen-detected and symptomatic cases

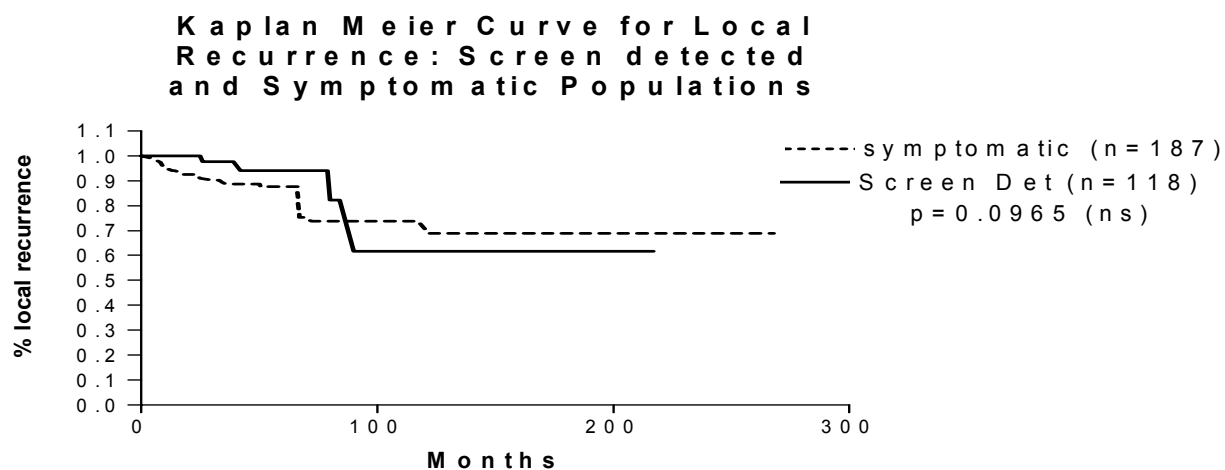


Fig.Appendix 5. Regional recurrence in screen-detected and symptomatic cases

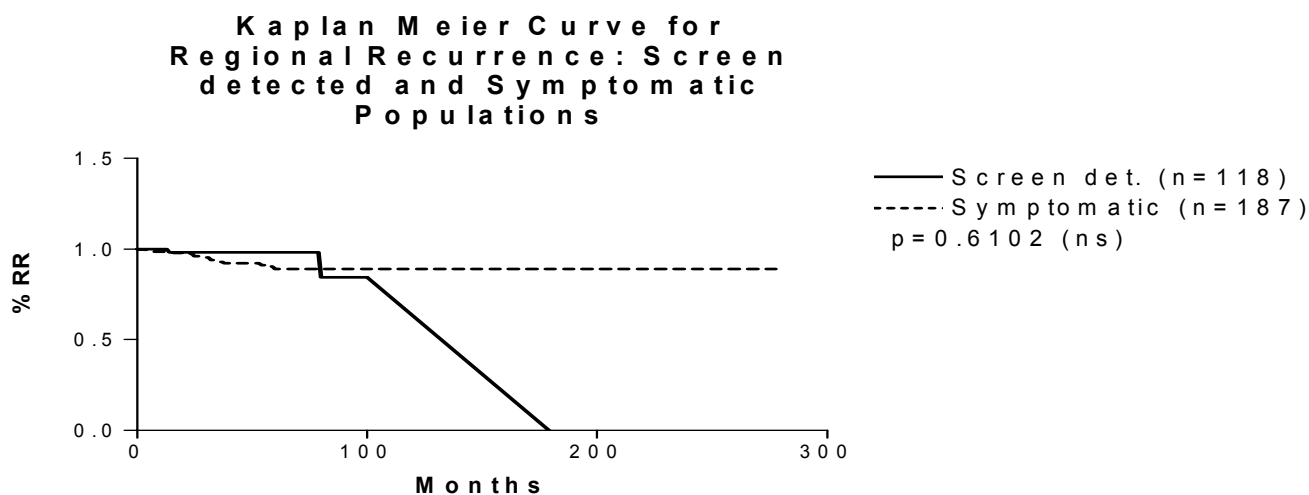
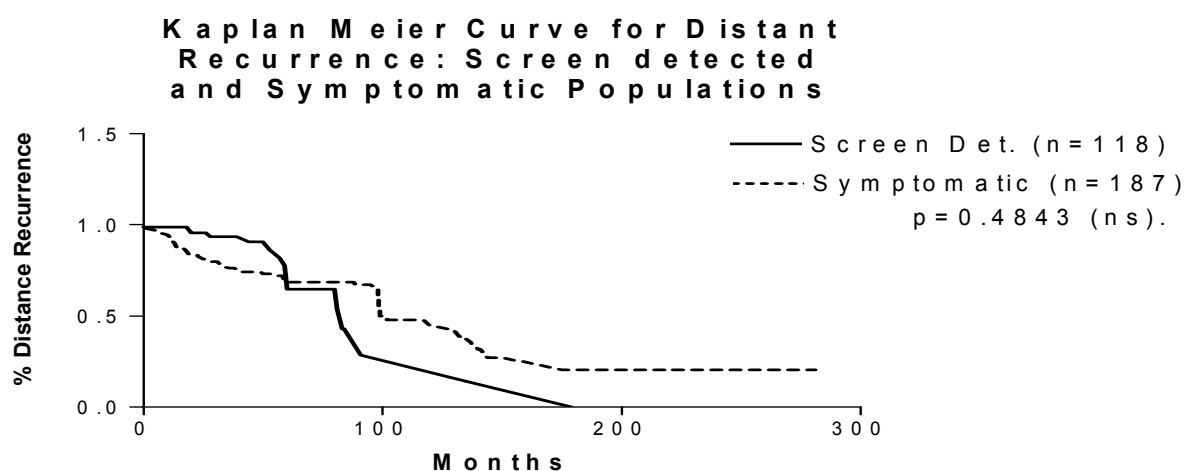
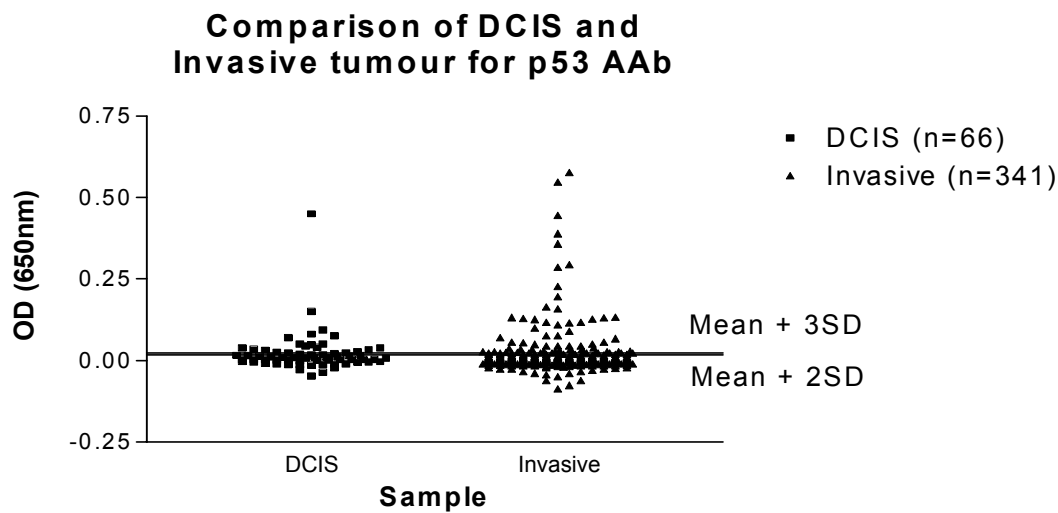


Fig.Appendix 6. Distant recurrence in screen-detected and symptomatic cases



Assessing the signal difference between pre and invasive tumours for the 3 tumour markers.

Fig. Appendix 7. Comparison of DCIS and Invasive cases for p53 AAb



Median OD for DCIS: 0.009500 (Range -0.04867- 0.4497)

Median OD for Invasive Tumour: 0.002333 (Range -0.08733- 0.5770)

P = 0.0398 (Non-parametric Mann-Whitney test for significance)

For MUC1 and c-myc AAbs, there was no significant difference in median signals between the pre-invasive and invasive populations ( $p = 0.665$  and  $p = 0.435$  respectively)

Comparison of panel positivity for DCIS versus Control and at-risk groups.

Table Appendix 1. Comparison of DCIS and Control Groups for panel positive cases

	<b>Control</b>	<b>DCIS</b>	<b>Total</b>
Panel +ve	10	29	39
Panel -ve	100	37	137
Total	110	66	176

Fishers exact test for significance

$P < 0.0001$  (significant)

Table Appendix 2. Comparison of DCIS and At-risk Groups for panel positive cases

	<b>At-Risk</b>	<b>DCIS</b>	<b>Total</b>
Panel +ve	70	29	99
Panel -ve	311	37	348
Total	381	66	447

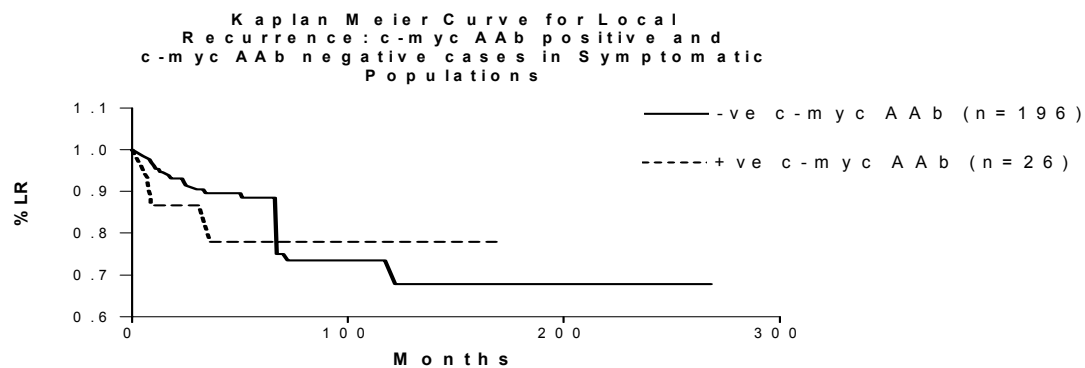
Fishers exact test for significance

$P < 0.0001$  (significant)



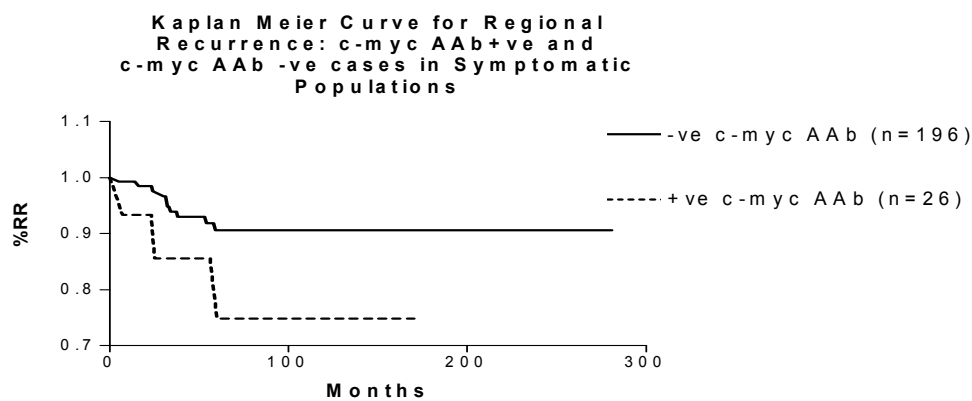
Comparison of c-myc AAb -ve and +ve groups for recurrence in symptomatic population.

Fig. Appendix 8. Local recurrence in c-myc AAb –ve and +ve groups



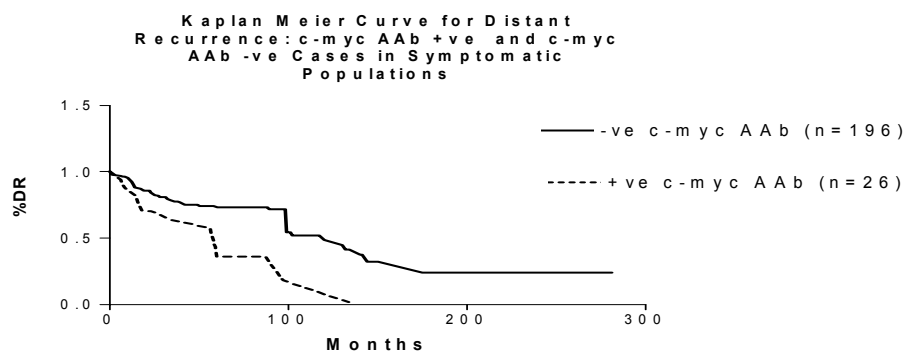
Logrank test for significance  $p = 0.7565$  (Non Significant).

Fig. Appendix 9. Regional recurrence in c-myc AAb –ve and +ve groups



Logrank test for significance  $p = 0.0976$  (Non Significant).

Fig. Appendix 10. Distant recurrence in c-myc AAb –ve and +ve groups



Logrank test for significance  $p = 0.0016$  (Significant).

Assessing the impact on sensitivity and specificity of using alternative panels or increasing the cut-off value to Mean+4SD

Table Appendix 3. Sensitivities and specificities of various panel combinations and higher cut-off value

<b>Panel</b>	<b>Screening Sensitivity N (%)</b>	<b>PBC Sensitivity N (%)</b>	<b>Specificity</b>
p53 + c-myc AAbs	1 (7.7%)	118 (29%)	93.6%
p53 + MUC1 AAbs	2 (15.4%)	116 (28.5%)	94.5%
c-myc + MUC1 AAbs	1 (7.7%)	74 (18.1%)	92.7%
Mean + 4SD cut-off value	2 (15.4%)	84 (20.6%)	97.2%

## ATAC TRIAL

ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial stopped recruiting in 2000. It is a large phase 3 double blind randomised controlled clinical trial which compared anastrozole with tamoxifen for post menopausal women who had early stage breast cancer. The trial involved over 9,000 post menopausal women with early stage breast cancer from all over the world. After these women had surgery for their breast cancer, they took either 5 years of tamoxifen or 5 years of anastrozole as part of the trial. The hormone therapy was part of their adjuvant treatment.

### Inclusion criteria:

- Postmenopausal women with histologically proven operable breast cancer.
- They had completed their primary surgical or chemotherapy treatment.
- Considered candidates for adjuvant endocrine treatment.

### Exclusion criteria:

- Evidence of metastatic disease.
- A gap of 8 weeks or more between primary treatment and randomisation.
- Previous endocrine therapy use.

### Primary end point:

- Disease Free Survival.
- New primary PBC.
- Death due to any cause.